



NOD2 NUCLEIC ACIDS AND PROTEINS

5 This application claims priority to U.S. provisional patent application serial number 60/244,289, which is herein incorporated by reference in its entirety. This patent application was supported in part by grant CA-64556 from the National Institutes of Health. The government has certain rights in the invention.

FIELD OF THE INVENTION

10 The present invention relates to intracellular signaling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The present invention provides assays for the detection of Nod2 and Nod2 polymorphisms associated with disease states. The present invention further provides inhibitors of Nod2 signaling and methods for identifying Nod2 pathway components.

BACKGROUND OF THE INVENTION

15 Inflammatory bowel diseases (IBD) are defined by chronic, relapsing intestinal inflammation of obscure origin. IBD refers to two distinct disorders, Crohn's disease and ulcerative colitis (UC). Both diseases appear to involve either a dysregulated immune response to GI tract antigens, a mucosal barrier breach, and/or an adverse inflammatory reaction to a persistent intestinal infection. The GI tract luminal contents and bacteria constantly stimulate the mucosal immune system, and a delicate balance of proinflammatory and anti-inflammatory cells and molecules maintains the integrity of the GI tract, without eliciting severe and damaging inflammation. It is unknown how the IBD inflammatory cascade begins, but constant GI antigen-dependent stimulation of the mucosal and systemic immune systems perpetuates the inflammatory cascade and drives lesion formation.

20 There is no known cure for IBD, which afflicts 2 million Americans. Current methods of managing IBD symptoms cost an estimated \$1.2 billion annually in the United States alone.

25 In patients with IBD, ulcers and inflammation of the inner lining of the intestines lead to symptoms of abdominal pain, diarrhea, and rectal bleeding.

Ulcerative colitis occurs in the large intestine, while in Crohn's, the disease can involve the entire GI tract as well as the small and large intestines. For most patients, IBD is a chronic condition with symptoms lasting for months to years. It is most common in young adults, but can occur at any age. It is found worldwide, but is most common in industrialized countries such as the United States, England, and northern Europe. It is especially common in people of Jewish descent and has racial differences in incidence as well. The clinical symptoms of IBD are intermittent rectal bleeding, crampy abdominal pain, weight loss and diarrhea. Diagnosis of IBD is based on the clinical symptoms, the use of a barium enema, but direct visualization (sigmoidoscopy or colonoscopy) is the most accurate test. Protracted IBD is a risk factor for colon cancer. The risk for cancer begins to rise significantly after eight to ten years of IBD.

Some patients with UC only have disease in the rectum (proctitis). Others with UC have disease limited to the rectum and the adjacent left colon (proctosigmoiditis). Yet others have UC of the entire colon (universal IBD). Symptoms of UC are generally more severe with more extensive disease (larger portion of the colon involved with disease).

The prognosis for patients with disease limited to the rectum (proctitis) or UC limited to the end of the left colon (proctosigmoiditis) is better than that of full colon UC. Brief periodic treatments using oral medications or enemas may be sufficient. In those with more extensive disease, blood loss from the inflamed intestines can lead to anemia, and may require treatment with iron supplements or even blood transfusions. Rarely, the colon can acutely dilate to a large size when the inflammation becomes very severe. This condition is called toxic megacolon. Patients with toxic megacolon are extremely ill with fever, abdominal pain and distention, dehydration, and malnutrition. Unless the patient improves rapidly with medication, surgery is usually necessary to prevent colon rupture.

Crohn's disease can occur in all regions of the gastrointestinal tract. With this disease intestinal obstruction due to inflammation and fibrosis occurs in a large number of patients. Granulomas and fistula formation are frequent complications of Crohn's

disease. Disease progression consequences include intravenous feeding, surgery and colostomy.

The most commonly used medications to treat IBD are anti-inflammatory drugs such as the salicylates. The salicylate preparations have been effective in treating mild to moderate disease. They can also decrease the frequency of disease flares when the medications are taken on a prolonged basis. Examples of salicylates include sulfasalazine, olsalazine, and mesalamine. All of these medications are given orally in high doses for maximal therapeutic benefit. These medicines are not without side effects. Azulfidine can cause upset stomach when taken in high doses, and rare cases of mild kidney inflammation have been reported with some salicylate preparations.

Corticosteroids are more potent and faster-acting than salicylates in the treatment of IBD, but potentially serious side effects limit the use of corticosteroids to patients with more severe disease. Side effects of corticosteroids usually occur with long term use. They include thinning of the bone and skin, infections, diabetes, muscle wasting, rounding of faces, psychiatric disturbances, and, on rare occasions, destruction of hip joints.

In IBD patients that do not respond to salicylates or corticosteroids, medications that suppress the immune system are used. Examples of immunosuppressants include azathioprine and 6-mercaptopurine. Immunosuppressants used in this situation help to control IBD and allow gradual reduction or elimination of corticosteroids. However, immunosuppressants cause increased risk of infection, renal insufficiency, and the need for hospitalization.

Clearly there is a great need for identification of the molecular basis of IBD, or its associated disorders Crohn's disease and ulcerative colitis.

SUMMARY OF THE INVENTION

The present invention relates to intracellular signaling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The present invention provides assays for the detection of Nod2 and Nod2 polymorphisms associated with

disease states. The present invention further provides inhibitors of Nod2 signaling and methods for identifying Nod2 pathway components.

Thus, in some embodiments, the present invention provides an isolated and purified nucleic acid comprising a sequence encoding a protein selected from the group consisting of SEQ ID NOs: 2,3 and 34. In some embodiments, the nucleic acid sequence is operably linked to a heterologous promoter. In some embodiments, the nucleic acid sequence is contained within a vector. In some further embodiments, the vector is within a host cell.

In other embodiments, the present invention provides an isolated and purified nucleic acid sequence that hybridizes under conditions of low stringency to a nucleic acid selected from the group consisting of SEQ ID NO:1 and 33. In some embodiments, the nucleic acid sequence encodes a protein that activates NF- κ B. In other embodiments, the present invention provides a vector comprising the nucleic acid sequence. In still other embodiments, the vector is within a host cell. In some embodiments, the host cell is located in an organism selected from the group consisting of a plant and an animal.

In yet other embodiments the present invention provides a protein encoded by a nucleic acid selected from the group consisting of SEQ ID NOs:1 and 33 and variants thereof that are at least 80% identical to SEQ ID NOs: 1 and 33, wherein the protein has at least one activity of Nod2. In some embodiments, the activity is activation of NF- κ B. In other embodiments, the activity is binding to RICK. In some embodiments, the protein is at least 90% identical to SEQ ID NOs:1 and 33. In other embodiments, the protein is at least 95% identical to SEQ ID NOs:1 and 33.

In still further embodiments, the present invention provides a method for producing variants of Nod2 comprising: providing a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 and 33; mutagenizing the nucleic acid sequence; and screening the variant for Nod2 activity.

In additional embodiments, the present invention provides a nucleic acid encoding Nod2, wherein the Nod2 competes for binding to NF- κ B with a protein

encoded by a nucleic acid sequence selected from the group consisting of SEQ ID NOs:1 and 33.

In other embodiments, the present invention provides a composition comprising a nucleic acid that inhibits the binding of at least a portion of a nucleic acid selected from the group consisting of SEQ ID NOs:1 and 33 to their complementary sequences. In yet other embodiments, the present invention provides a polynucleotide sequence comprising at least fifteen nucleotides capable of hybridizing under stringent conditions to the isolated nucleotide sequence selected from the group consisting of SEQ ID NOs:1 and 33.

The present invention also provides a method for detection of a polynucleotide encoding Nod2 protein in a biological sample suspected of containing a polynucleotide encoding Nod2. The method includes hybridizing the polynucleotide sequence selected from the group consisting of SEQ ID NOs:1 and 33 and variants thereof that are at least 80% identical to SEQ ID NOs: 1 and 33 (and wherein the protein has at least one activity of Nod2) to the nucleic acid of the biological sample to produce a hybridization complex. In some embodiments, the method further includes the step of detecting the hybridization complex, wherein the presence of the hybridization complex indicates the presence of a polynucleotide encoding Nod2 in the biological sample. In some embodiments, prior to hybridization, the nucleic acid of the biological sample is amplified.

The present invention further provides a method for screening compounds for the ability to alter Nod2 activity, comprising: providing: a first polypeptide sequence comprising at least a portion of Nod2; ii) a second polypeptide sequence comprising at least a portion of a protein known to interact with Nod2; and iii) one or more test compounds; combining in any order, the first polypeptide sequence comprising at least a portion of Nod2, the second polypeptide sequence comprising at least a portion of a protein known to interact with Nod2, and one or more test compounds under conditions such that the first polypeptide sequence, the second polypeptide sequence, and the test compound interact; and detecting the presence or absence of an interaction between the polypeptide sequence comprising at least a portion of Nod2 and the

polypeptide sequence comprising at least a portion of a protein known to interact with Nod2. In some embodiments, the first polypeptide sequence is selected from the group consisting of SEQ ID NOs: 2-17 and 34. In some embodiments, the second polypeptide comprises RICK.

5 The present invention also provides a method of identifying individuals suffering from Crohn's disease or at risk of developing Crohn's disease comprising: providing nucleic acid from a patient; wherein the nucleic acid comprises a Nod2 allele; and detecting a mutation in the nucleic acid, wherein the mutation results in increased NF- κ B activation. In some embodiments, the mutation is in said Nod2
10 allele. In some embodiments, the mutation is a cytosine residue insertion. In still further embodiments, the mutation causes a deletion of at least one LRR repeat of Nod2. In some embodiments, the detecting step is accomplished by hybridization analysis. In some embodiments, the method further includes the step of providing a prognosis to the patient based on the presence or absence of the mutation.

15 In yet other embodiments, the present invention provides a kit for determining if a subject is at risk of developing Crohn's disease comprising: at least one reagent that specifically detects a mutation in a Nod2 allele; and instructions for determining that the subject is at increased risk of developing Crohn's disease.

20 The present invention also provides a purified polypeptide selected from the group consisting of SEQ ID NOs:2, 3, and 34.

The present invention additionally provides a compound capable of inhibiting the binding of a Nod2 to a RICK polypeptide.

DESCRIPTION OF THE FIGURES

25 Figure 1 shows the deduced Amino Acid Sequence and Domain Structure of Human Nod2. Figure 1A shows the amino acid sequence of Nod2 (SEQ ID NO:4). Caspase recruitment domains (CARD 1 and 2; SEQ ID NOs: 5 and 6), nucleotide binding domain (NBD; SEQ ID NO:7) and leucine-rich repeats (LRRS; SEQ ID NOs:8-17) are indicated by reverse highlight, underline and arrows, respectively. The

consensus sequence of the P-loop (Walker A box; SEQ ID NO:18) and the Mg²⁺ binding site (Walker B box; SEQ ID NO:19) are indicated by boxes. Figure 1B shows the domain structure of Nod2. Numbers corresponds to amino acid residues shown in panel A. The region homologous to the CARDS, NBD and LRRs are indicated by black closed, dark closed, and hatched boxes, respectively.

Figure 2 shows an alignment of Human Nod2 and Related Proteins. Figure 1A shows an alignment of CARDS of Nod2 (SEQ ID NOs:5 and 6), Nod1 (GeneBank accession number AF113925; SEQ ID NO:20), RICK (AF027706; SEQ ID NO:21), ARC (AF043244; SEQ ID NO:22), RAIDD (U79115; SEQ ID NO:23), Caspase-2 (U13021; SEQ ID NO:24), Ced-3 (L29052; SEQ ID NO:25), Ced-4 (X69016; SEQ ID NO:26), Caspase-9 (U56390; SEQ ID NO:27), Apaf-I (AF013263; SEQ ID NO:28) and c-IAP-1 (L49431; SEQ ID NO:29). Hydrophobic residues are shown in reverse highlighting. Negatively and positively charged residues are highlighted in light and dark gray, respectively. Proline and glycine residues (($\alpha\beta$ breaker) are bolded. The putative (α helices, H1 to H5, are shown according to the three dimensional structure of the CARD of RAIDD (Chou *et al.*, Cell, 94:171 [1998])). Figure 2B shows an alignment of NBDs of Nod2 (SEQ ID NO:7), Nod1 (SEQ ID NO:30), Apaf-I (SEQ ID NO:31) and Ced-4 (SEQ ID NO:32). The residues identical and similar to those of Nod2 are shown by reverse and dark highlighting, respectively. The consensus sequence of the P-loop (Walker A box) and the Mg²⁺ binding site (Walker B box) are indicated by boxes. The residues identical and similar to those of Nod2 are shown by reverse and dark highlighting, respectively. Figure 2C shows an alignment of LRRs of Nod2 (SEQ ID NOs: 8-17). The conserved positions with leucine and other hydrophobic residues are indicated by dark and light gray highlighting, respectively. The putative (α helix and β sheet are shown according to the three dimensional structure of the ribonuclease inhibitor (Kobe and Deisenhofer, Curr. Opin. Struct Biol., 5:409-416 [1995])).

Figure 3 shows an expression Analysis of Nod2. Figure 3A shows a northern blot analysis of nod2 expression in human tissues; PBL (peripheral blood leukocytes). Figure 3B shows RT-PCR analysis of nod2 expression in granulocyte, monocyte and

lymphocyte enriched populations. Two sets of Nod2 oligonucleotide primers (P1-P2 and P3-P4) were used to amplify the nucleotide sequences of the CARDs and LRRs, respectively. Figure 3C shows the nucleotide sequence of the 5' region of *nod2*. Two potential in-frame translation initiation sites separated by 81 nucleotides are indicated by arrows. Figure 3D shows immunoblotting of *nod2* gene products expressed in HEK293T cells. Cells were transfected with control plasmid (lane 1), or constructs containing both potential translation initiator sites of Nod2 (lane 2), or as a control the second translation initiation site corresponding to that of Nod2b (lane 3) or the most NH₂-terminal translation initiation site (lane 4) in the context of a canonical translation initiation motif. In all cases, a Nod2 protein lacking residues 302-1040 and HA tagged at its COOH terminus was expressed to facilitate detection of *nod2* gene products. Nod2 proteins were detected by immunoblotting with anti-HA antibody and indicated by a and b.

Figure 4 shows mutational Analysis of Nod2. Figure 4A shows wt and mutant Nod2 proteins. CARDs, NBD and LRRs are indicated by black closed, dark closed, and hatched boxes, respectively. Numbers represent amino acid residue in Nod2 protein. Figure 4B shows expression analysis of wt and mutant Nod2 proteins. HEK293T cells were transfected with control plasmid (-) or 5 µg of plasmids producing the indicated HA-tagged Nod2 proteins. Extracts from equal number of cells were immunoprecipitated with rabbit anti-HA antibody and immunoblotted with mouse monoclonal anti-HA antibody. The expected size of CARDs, CARD1 and LRRs mutant proteins are indicated by black arrowheads. Figure 4C shows NF-κB Activation by Nod2 proteins. Induction of NF-κB activation was determined from triplicate culture of HEK293T cells co-transfected with the indicated amount of wt or mutant Nod2 expression plasmids in the presence of pBVIx-Luc and pEF-BOS-β-gal as described below. Values represent mean ± SD of triplicate cultures.

Figure 5 shows that Nod2 Acts through the IKK complex to activate NF-KB. Figure 5A shows inhibition of Nod2 and TNFα-induced NF-KB activation by dominant negative mutant proteins of the NF-κB pathway. Induction of NF-κB activation was determined in triplicate cultures of HEK293T cells transfected with 30

ng of Nod2 plasmid (open bars) or stimulated with 10 ng/ml of TNF α for 4 h (closed bars) and 70 ng of I- κ B α S32A/S36A, IKK α K44A, IKK β K44A, RICK (406-540) or RIP (558-671) expression plasmid in the presence of pBVix-Luc and pEF-BOS- β -gal. Results are presented as a percent of values obtained with Nod2 and control plasmid.

In the experiment shown, Nod2 and TNF α induced 58 ± 8 -fold and 14 ± 1 -fold activation of NF- κ B, respectively. Values represent mean \pm SD of triplicate cultures. Figure 5B shows induction of NF- κ B in parental Rat-1 and derivative 5R cells. Induction of NF- κ B activation was determined from triplicate cultures of 1×10^5 HEK293T cells co-transfected with the indicated plasmids and pBVix-Luc in the presence of control plasmid pEF-BOS- β -gal. Values represent mean \pm SD of triplicate cultures.

Figure 6 shows the interaction of Nod2 with RICK. Figures 6A and B show the interaction between wt and mutant Nod2 with RICK. HEK293T cells were co-transfected with wt or mutant Nod2 and RICK expression plasmid. The co-immunoprecipitated RICK was detected by immunoblotting with anti-Flag antibody (upper panel). Nod2 immunoprecipitates are shown in lower panel. Total lysates were blotted with anti-Flag antibody (middle panel). Figure 6C shows the interaction between Nod2 and wt and mutant RICK. HEK293T cells were co-transfected with wt Nod2 and wt or mutant RICK- Δ CARD (residues 1-374) or RICK-CARD (residues 374-540) expression plasmid. The co-immunoprecipitated Nod2 was detected by immunoblotting with anti-HA antibody (upper panel). Total lysates were blotted with anti-Flag (middle panel) or anti-HA (lower panel) antibody. A background band is shown by asterisk.

Figure 7 shows that enforced oligomerization of Nod2 induces NF- κ B activation. Figure 7A shows an expression analysis of wt and mutant Fpk3-Nod2 chimeric proteins. HEK293T cells were transfected with of control plasmid (-) or plasmids producing the indicated Myc-tagged Fpk3Nod2 proteins. Extracts from equal number of cells was immunoprecipitated and immunoblotted with rabbit anti-Myc antibody. Figure 7B shows that enforced oligomerization of Nod2 induces NF- κ B activation. 2×10^5 HEK293T cells were transfected with 1 ng of the indicated

plasmids in the presence of pBVlx-luc and pEF-BOS- β -gal. 8 hr post-transfection, cells were treated with 500 nM AP1510 (black bars) or left untreated (white bars). 24 hr post-transfection, the κ B-dependent transcription was determined as described below. Values represent mean \pm SD of triplicate cultures.

Figure 8 shows the response of HEK293T cells expressing Nod1 to bacterial and fungal pathogen components. Figure 8A shows data from 1×10^5 HEK293T cells that were transfected with 0.3 ng of pcDNA3-Flag (white bars) or pcDNA3Nod1-Flag (black bars) in the presence of 600 ng of pcDNA3, 73 ng pEFIBOS- β gal and 7.3 ng pBXIV-luc. 8 hr post-transfection, cells were treated with 10 μ g/ml of each pathogen product, lipoteichoic acid (LTA) or peptidoglycan (PGN) from *Staphylococcus aureus*, lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, mannan from *Candida albicans* 20A, synthetic soluble bacterial lipoprotein (SBLP) or left untreated (Control). 24 hr post-transfection, κ B-dependent transcription was determined by luciferase activity relative and values normalized to β -galactosidase in triplicate cultures. As control, the inset showed Nod1 proteins immunodetected with anti-FLAG Ab in lysates from cells transfected with 10 ng pcDNA3-Nod1 in presence (right) and absence (left) of 10 μ g/ml LPS. Figure 8B shows data from 1×10^5 HEK293T cells that were transfected with 0.3 ng of pcDNA3-Flag (-), pcDNA3-Nod1 -Flag (Nod1) or pcDNA3-Nod1(I-648)-Flag (Nod1 Δ LRR), 300 ng pcDNA3-FLAG-TLR4, 3 ng pCMV-IL1R1 plus 100 ng pcDNA3-IL1 β -HA (IL1) or 1 ng pcDNA3-RIP-Flag (RIP) in the presence of 600 ng of pcDNA3, 73 ng pEF 1BOS- β gal and 7.3 ng pBXIV-luc. Eight hr post-transfection, cells were treated with 10 μ g/n-A LPS (black bars) or left untreated (white bars). Twenty-four hr post-transfection, κ B-dependent transcription was determined as described above.

Figure 9 shows differential responsiveness of Nod1 and Nod2 to LPS from various sources. 1×10^5 HEK293T cells were transfected with 0.3 ng of pcDNA3-Flag (-), pcDNA3-Nod1-Flag (Nod1) or pcDNA3-Nod1(1-648)-Flag (Nod1 Δ LRR), 0.03ng of pcDNA3-Nod2 or pcDNA3-Nod2(1-744)-Flag (Nod2 Δ LRR) in the presence of 600 ng of pcDNA3, 73 ng pEF1BOS- β gal and 7.3 ng pBXIV-luc. 8 hr post-transfection, cells were treated with 10 μ g/ml each pathogen, LTA from *S.*

aureus or *S. sanguis*, PGN from *S. aureus*, LPS from *Pseudomonas aeruginosa*, *Shigella flexneri* 1A, *Serratia marcescens*, *Salmonella typhimurium*, *Klebsiella pneumoniae* or *E. coli* 055:B5, or left alone without treatment. For TNF α stimulation, 22 hrs after transfection, cells were incubated with 10 ng/ml of TNF α for 2 hr.

5 Figure 10 shows the physical Interaction between Nod1 and LPS. 1×10^8 HEK293T cells were transfected with 30 μ g of pcDNA3-Flag-Nod1, pRK7-FLAG-IKK β , pcDNA3-FLAG-IKK γ , pcDNA3-FLAG-IKK α or pcDNA3-CIPER-FLAG (Takeuchi *et al.*, Immunity, 4:443 [1999]). 24 hr post-transfection, S100 fractions were prepared from transfected cells as described below. The radioactivity of [3 H] LPS co-immunoprecipitated with anti-FLAG Ab was determined as described below. Figure 10A shows S100 lysate from transfected cells was incubated with [3 H] LPS, anti-FLAG M2 Ab, Protein A-Sepharose and Protein G-Sepharose. Figure 10B shows data for proteins that were immunopurified first from 20 mg of S100 lysate and incubated with [3 H] LPS in the presence of 10 mg BSA. The co-immunoprecipitated radioactivity was determined as described in detail below. Expression of each protein in 50 μ g of S100 lysate was immunodetected with anti-FLAG Ab.

Figure 11 shows the nucleic acid sequence of SEQ ID NO:33.

Figure 12 shows the nucleic acid sequence of SEQ ID NO:1.

Figure 13 shows the polypeptide sequence of SEQ ID NO:2.

Figure 14 shows the polypeptide sequence of SEQ ID NO:3.

Figure 15 shows the polypeptide sequence of SEQ ID NO:34.

Figure 16 shows the nucleic acid (SEQ ID NOs: 35 (wild type) and 36 (mutant)) and polypeptide (SEQ ID NO:51 (wild type) and SEQ ID NO:52 (mutant)) of Nod2 Exon 11.

GENERAL DESCRIPTION OF THE INVENTION

The present invention relates to intracellular signalling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The Nod2 protein was found to have structural homology to the Nod1 protein. Apaf-1 and Nod1 (also called

CARD4) are members of a family of intracellular proteins that are composed of an NH2-terminal caspase-recruitment domain (CARD), a centrally located nucleotide-binding domain (NBD) and a COOH-terminal regulatory domain (Bertin *et al.*, J. Biol. Chem. 274: 12955-12958 [1999], Inohara *et al.*, J. Biol. Chem. 274: 14560-14568 [1999]). While Apaf-1 possesses WD40 repeats, Nod1 contains leucine-rich repeats (LRRs) in its C-terminus. The structural and functional similarities between Apaf-1 and Nod1 suggest that these proteins share a common molecular mechanism for activation and effector function. In the case of Apaf-1, the WD-40 repeats act as a recognition domain for mitochondrial damage through binding to cytochrome c, allowing Apaf-1 to oligomerize and interact with procaspase-9 through a CARD-CARD homophilic interaction (Li *et al.*, Cell 91: 479-489 [1997], Zou *et al.*, J. Bio. Chem. 274: 11549-11556 [1999]). Apaf-1 oligomerization is mediated by the NBD and is thought to induce the proximity and proteolytic activation of procaspase-9 molecules in the apoptosome complex (Srinivasula *et al.*, Mol. Cell 1: 949-957 [1998], Hu *et al.*, J. Bio. Chem. 273: 33489-34494 [1998]).

Previous studies showed that Nod1 promotes apoptosis when overexpressed in cells, but unlike Apaf-1, it induces NF- κ B activation (Bertin *et al.*, *supra*, Inohara *et al.*, *supra*). NF- κ B activation induced by Nod1 is mediated by the association of the CARD of Nod1 with the corresponding CARD of RICK (also called RIP2 and CARDIAK), a protein kinase that activates NF- κ B (Bertin *et al.*, *supra*, Inohara *et al.*, *supra*, Inohara *et al.*, J. Biol. Chem. 273: 12296-12300 [1998], McCarthy *et al.*, J. Bio. Chem. 273, 16968-16975 [1998], Thome *et al.*, Curr. Biol. 8: 885-888 [1998]). Analyses with wild-type (wt) and mutant forms of both Nod1 and RICK have suggested that Nod1 and RICK act in the same pathway of NF- κ B activation, where RICK functions as a downstream mediator of Nod1 signaling (Bertin *et al.*, *supra*, Inohara *et al.*, [1999] *supra*, Inohara *et al.*, J. Biol. Chem. 275: 27823-27831 [2000]). Nod1 self-associates through its NBD and Nod1 oligomerization promotes proximity of RICK molecules and NF- κ B activation (Inohara *et al.*, [2000], *supra*). Nod1 also displays striking similarity to a class of disease resistance (R) proteins found in plants

(Parniske *et al.*, Cell 91: 821-832 [1997], Dixon *et al.*, Proc. Natl. Acad. Sci. U. S. A. 97: 8807-8814 [2000]). Like Nod1, these intracellular R proteins contain N-terminal effector domains linked to a NBD and share with Nod1 the presence of multiple LRRs located C-terminally of the NBD (Bertin *et al.*, *supra*, Dixon *et al.*, *supra*). After
5 specific recognition of pathogen products, these R proteins mediate a defense response associated with metabolic alterations and localized cell death at the site of pathogen invasion (Dixon *et al.*, *supra*). The LRRs of R proteins are highly diverse and appear to be involved in the recognition of a wide array of pathogen components (Parniske *et al.*, *supra*, Dixon *et al.*, *supra*). The binding partner of the LRRs of Nod1 remains
10 unknown. The structural homology of Nod1 with plant R proteins suggest that other LRR-containing Nod1-like molecules may exist in the human genome to allow activation of these molecules by different sets of intracellular stimuli.

The identification and characterization of Nod2, a LRR-containing protein with structural and functional similarity to Nod1 is disclosed herein. These studies indicate
15 that Nod2 activates NF- κ B, but unlike Nod1, this new homologue is primarily expressed in monocytes. The present invention is not limited to any particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the present invention. Nevertheless, Nod2 is a member of the Nod1/Apaf-I family that activates NF- κ B through interactions with its NH₂-terminal
20 CARDS, as these domains were necessary and sufficient for NF- κ B activation. Nod2 associated with RICK via a homophilic CARD-CARD interaction. The NF- κ B-inducing activity of Nod2 correlated with its ability to associate with RICK and was inhibited by a RICK mutant, suggesting that RICK is a direct downstream target of Nod2. Thus, the signaling pathways of both Nod1 and Nod2 appear to utilize
25 RICK as a downstream mediator of NF- κ B activation. In contrast to Nod1, two tandem CARDS are present in the NH₂-terminus of Nod2 and both were required for association with RICK and NF- κ B activation.

Nod2 is the first molecule known to contain two CARDS. The molecular basis underlying the requirement of both CARDS of Nod2 for RICK binding remains

unclear. The present invention is not limited to any particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the present invention. Nevertheless, it is contemplated that the presence of both CARDs may enhance the affinity for the CARD of RICK. Another possibility is that upon an initial interaction involving a CARD of Nod2 and the CARD of RICK, Nod2 may undergo a conformational change that allows the second CARD to associate with high affinity to RICK. The intermediate region of RICK associates with IKK γ (Inohara *et al.*, [2000], *supra*), providing a direct link between Nod1/Nod2 and the IKK complex. Consistent with this model, NF- κ B activation induced by Nod2 as well as that induced by Nod1 required IKK γ and was inhibited by dominant negative forms of IKK γ , IKK α and IKK β . The functional role for the LRRs of Nod1 and Nod2 remains unclear. The LRR is a repeated protein-protein interaction module that is presumably involved in the activation of Nod1 and Nod2 by upstream signals. In the case of plant NBD/LRR-containing R proteins, their LRRs appear to be important for the recognition of pathogen components and their N-terminal domains appear to mediate a signaling cascade that regulates gene expression (Parniske *et al.*, *supra*, Dixon *et al.*, *supra*). Because both Nod1 and Nod2 activate NF- κ B, their LRRs may act to recognize a different set of intracellular stimuli that mediate Nod1 and Nod2 oligomerization and association with RICK. Because Nod2 is expressed primarily in monocytes, Nod2 might serve as an intracellular receptor that transduces signals in the monocyte/macrophage that lead to activation of NF- κ B and transcription of regulatory genes.

The Nod2 proteins of the present invention are also involved in the recognition of microbial pathogens. The innate immune system regulates the immediate response to microbial pathogens in multiple organisms including humans. The innate immune response is initiated by recognition of specific pathogen components by host immune cells. Mammalian cells have cell surface receptors and intracellular mechanisms that initiate the defense response against microbial pathogens (Aderem and Ulevitch, *Nature*, 406:785-787 [2000]; Philpott *et al.*, *J. Immunol.*, 165:903-914 [2000]). Toll

like receptors (TLRs) comprise a family of cell surface receptors that are related to the *Drosophila* Toll protein, a molecule involved in defense against fungal infection in the fly (Aderem and Ulevitch, *Supra*). Ten mammalian TLRs have been identified (Aderem and Ulevitch, *Supra*). Two members of the family, TLR2 and TLR4, have
5 been better characterized and shown to mediate the response to multiple bacterial cell-wall components including lipopolysaccharide (LPS), lipopeptides, peptidoglycans (PGN) and lipoteichoic acid (LTA) (Yang *et al.*, *Nature*, 395:284-288 [1998]; Poltorak *et al.*, *Science*, 282:2085-2088 [1998]; Aliprantis *et al.*, *Science*, 285:736-739 [1999]; Chow *et al.*, *J. Biol. Chem.*, 274:10689-10692 [2000]; and Schwandner *et al.*, *J. Biol.*
10 *Chem.*, 274: 17406-17409 [2000]). Mammalian TLRs have multiple leucine-rich repeats in the ectodomain and an intracellular Toll-IL1 receptor (TIR) domain that mediates a signaling cascade to the nucleus (Aderem and Ulevitch, *Supra*). Stimulation of TLR2 and TLR4 leads to the recruitment of the adaptor molecule MyD88 and the serine kinase IL-1R-associated kinase (IRAK), two signaling
15 components that together with TRAF-6 mediate activation of NF- κ B (Aderem and Ulevitch, *Supra*).

Plants have several classes of genes that regulate the defense against invading pathogens. An important class of these molecules is termed disease resistance (R) proteins, and members include both membrane-bound and cytosolic proteins. These
20 are essential for the defense against multiple pathogens including bacteria, fungi and viruses (Dixon *et al.*, *PNAS*, 97:8807-8814 [2000]). The cytosolic type of R proteins which include the Tobacco N gene product and up to 200 gene products in *Arabidopsis thaliana* are comprised of an N-terminal TIR or zinc finger effector domain, a centrally located nucleotide-binding domain (NBD) and C-terminal
25 leucine-rich repeats (LRRs) (Dixon *et al.*, *Supra*). The LRRs of cytosolic R proteins are highly diverse and appear to be involved in the recognition of a wide array of microbial components (Dixon *et al.*, *Supra*). This class of disease resistant proteins mediates the hypersensitive (HS) response in plants that includes metabolic alterations and localized cell death at the site of pathogen invasion (Dixon *et al.*, *Supra*). The

cytosolic R proteins of plants have remarkable structural homology to Nod1/CARD4, a recently described protein related to the apoptosis regulator Apaf-1 (Zou *et al.*, Cell, 90:405-413 [1997]; Bertin *et al.*, J. Biol. Chem., 274:12955-12958; and Inohara *et al.*, J. Biol. Chem., 274:14560-14568 [1999]). Like plant R proteins, Nod1 is comprised of an N-terminal effector domain, a centrally located NBD and multiple LRRs at the C-terminus (Bertin *et al.*, *Supra*; Inohara *et al.*, *Supra*). Nod1 induces NF- κ B activation which is mediated through the association of its N-terminal caspase-recruitment domain (CARD) with that of RICK, a protein kinase that also activates NF- κ B (Bertin *et al.*, *Supra*; Inohara *et al.*, *Supra*; Inohara *et al.*, J. Biol. Chem., 273:12296-12300 [1998]; McCarthy *et al.*, J. Biol. Chem., 273:16968-16975; Thome *et al.*, Curr. Biol., 8:885-888 [1998]; Inohara *et al.*, J. Biol. Chem., 275:27823-27831 [2000]). However, the trigger molecule(s) which activates Nod1 to mediate NF- κ B activation remains unknown.

The present invention also demonstrates that lipopolysaccharide (LPS) induces NF- κ B activation in HEK293T cell expressing Nod1, whereas parental HEK293T cells are insensitive to LPS. The present invention is not limited to a particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the present invention. Nevertheless, in the human system, the TLR4/MD2/CD14 complex has been demonstrated to serve as a surface receptor for LPS (Aderem and Ulevitch, *Supra*). In addition to the cell surface TLR4 complex, there is mounting evidence that mammalian cells have an intracellular receptor that detects LPS in the cytoplasm of bacteria infected cells (Philpott *et al.*, *Supra*). For example, epithelial cells are unresponsive to extracellular LPS either purified or presented in the context of non-invasive Gram negative bacterial strains (Philpott *et al.*, *Supra*). Yet, LPS introduced inside of the epithelial cells activates NF- κ B (Philpott *et al.*, *Supra*). However, to date, the identification of an intracellular recognition system for LPS and/or other microbial products remains elusive. Because Nod1 can confer responsiveness to LPS, Nod1 may act as an intracellular receptor for LPS. Nod1 function might be important in the intracellular response of epithelial cells

against invading bacteria, as Nod1 is expressed in intestinal, lung and nasal epithelial surfaces in the late mouse embryo (Inohara *et al.*, *Supra*). The presence of an intracellular detection system for bacterial LPS would be expected in epithelial surfaces such as those of the gut that are highly exposed to bacteria and bacterial products. In such organs, triggering of an inflammatory response to bacterial products through surface receptors such as TLR4 would be detrimental to the organism. HEK293T cells expressing Nod2, another member of Nod family, respond to LPS but Nod1 and Nod2 appear to have different preferences for LPS preparations from different bacteria. These observations suggest that in addition to TLRs, Nod family members may represent another innate immune system for the recognition of a wide array of pathogen products. For example, the genome of the plant *Arabidopsis thaliana* contains approximately 200 disease resistance genes encoding intracellular NBD-LRR proteins related to Nod1 and Nod2 (Dixon *et al.*, *Supra*).

Definitions

To facilitate understanding of the invention, a number of terms are defined below.

As used herein, the term "Nod2" when used in reference to a protein or nucleic acid refers to a protein or nucleic acid encoding a protein that, in its wild type form, activates NF- κ B and contains two CARDs (caspase recruitment domains). The term Nod2 encompasses both proteins that are identical to wild-type Nod2 and those that are derived from wild type Nod2 (*e.g.*, variants of Nod2 or chimeric genes constructed with portions of Nod2 coding regions).

As used herein, the term "activates NF- κ B," when used in reference to any molecule that activates NF- κ B, refers to a molecule (*e.g.*, a protein) that induces the activity of the NF- κ B transcription factor through a cell signaling pathway. Assays for determining if a molecule activates NF- κ B utilize, for example, NF- κ B responsive reporter gene constructs. Suitable assays include, but are not limited to, those described in Examples 4 and 5.

As used herein, the term "activity of Nod2" refers to any activity of wild type Nod2. The term is intended to encompass all activities of Nod2 (*e.g.*, including, but not limited to, activating NF-kB, binding to RICK, and enhancing apoptosis).

The term "apoptosis" means non-necrotic cell death that takes place in metazoan animal cells following activation of an intrinsic cell suicide program. Apoptosis is a normal process in the development and homeostasis of metazoan animals. Apoptosis involves characteristic morphological and biochemical changes, including cell shrinkage, zeiosis, or blebbing, of the plasma membrane, and nuclear collapse and fragmentation of the nuclear chromatin, at intranucleosomal sites, due to activation of an endogenous nuclease.

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor (*e.g.*, Nod2). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' untranslated sequences. The sequences that are located 3' or downstream of the coding region and that are present on the mRNA are referred to as 3' untranslated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene that are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In particular, the term "Nod2 gene" refers to the full-length Nod2 nucleotide sequence (*e.g.*, contained in SEQ ID NO:1). However, it is also intended that the term encompass fragments of the Nod2 sequence, as well as other domains within the full-length Nod2 nucleotide sequence. Furthermore, the terms "Nod2 nucleotide sequence" or "Nod2 polynucleotide sequence" encompasses DNA, cDNA, and RNA (*e.g.*, mRNA) sequences.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences that are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences that direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product that has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the terms "modified", "mutant", and "variant" refer to a gene or gene product that displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of

deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

DNA molecules are said to have "5' ends" and "3' ends" because mononucleotides are reacted to make oligonucleotides or polynucleotides in a manner such that the 5' phosphate of one mononucleotide pentose ring is attached to the 3' oxygen of its neighbor in one direction via a phosphodiester linkage. Therefore, an end of an oligonucleotides or polynucleotide, referred to as the "5' end" if its 5' phosphate is not linked to the 3' oxygen of a mononucleotide pentose ring and as the "3' end" if its 3' oxygen is not linked to a 5' phosphate of a subsequent mononucleotide pentose ring. As used herein, a nucleic acid sequence, even if internal to a larger oligonucleotide or polynucleotide, also may be said to have 5' and 3' ends. In either a linear or circular DNA molecule, discrete elements are referred to as being "upstream" or 5' of the "downstream" or 3' elements. This terminology reflects the fact that transcription proceeds in a 5' to 3' fashion along the DNA strand. The promoter and enhancer elements that direct transcription of a linked gene are generally located 5' or upstream of the coding region. However, enhancer elements can exert their effect even when located 3' of the promoter element and the coding region. Transcription termination and polyadenylation signals are located 3' or downstream of the coding region.

As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or, in other words, the nucleic acid sequence that encodes a gene product. The coding region may be present in either a cDNA, genomic DNA, or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, *etc.* may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region

utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, *etc.* or a combination of both endogenous and exogenous control elements.

As used herein, the term "regulatory element" refers to a genetic element that controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element that facilitates the initiation of transcription of an operably linked coding region. Other regulatory elements include splicing signals, polyadenylation signals, termination signals, *etc.*

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (*i.e.*, identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid and is referred to using the functional term "substantially homologous." The term "inhibition of binding," when used in reference to nucleic acid binding, refers to inhibition of binding caused by competition of homologous sequences for binding to a target sequence. The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (*i.e.*, the hybridization) of a completely homologous to a target under conditions of low

stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (*i.e.*, selective) interaction. The absence of non-specific binding may be tested by the use of a second target that lacks even a partial degree of complementarity (*e.g.*, less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

The art knows well that numerous equivalent conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (*e.g.*, the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions that promote hybridization under conditions of high stringency (*e.g.*, increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.).

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

A gene may produce multiple RNA species that are generated by differential splicing of the primary RNA transcript. cDNAs that are splice variants of the same gene will contain regions of sequence identity or complete homology (representing the presence of the same exon or portion of the same exon on both cDNAs) and regions of complete non-identity (for example, representing the presence of exon "A" on cDNA 1 wherein cDNA 2 contains exon "B" instead). Because the two cDNAs contain regions of sequence identity they will both hybridize to a probe derived from the entire gene or portions of the gene containing sequences found on both cDNAs; the two splice variants are therefore substantially homologous to such a probe and to each other.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (*i.e.*, it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

As used herein, the term "competes for binding" is used in reference to a first polypeptide with an activity which binds to the same substrate as does a second polypeptide with an activity, where the second polypeptide is a variant of the first polypeptide or a related or dissimilar polypeptide. The efficiency (*e.g.*, kinetics or thermodynamics) of binding by the first polypeptide may be the same as or greater than or less than the efficiency substrate binding by the second polypeptide. For example, the equilibrium binding constant (K_D) for binding to the substrate may be different for the two polypeptides. The term " K_m " as used herein refers to the Michaelis-Menton constant for an enzyme and is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity in an enzyme catalyzed reaction.

As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (*i.e.*, the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the nucleic acids.

As used herein, the term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G + C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (*See e.g.*, Anderson and Young, Quantitative Filter Hybridization, *in Nucleic Acid Hybridization* [1985]). Other references include more sophisticated computations that

take structural as well as sequence characteristics into account for the calculation of T_m .

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. Those skilled in the art will recognize that "stringency" conditions may be altered by varying the parameters just described either individually or in concert. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences (*e.g.*, hybridization under "high stringency" conditions may occur between homologs with about 85-100% identity, preferably about 70-100% identity). With medium stringency conditions, nucleic acid base pairing will occur between nucleic acids with an intermediate frequency of complementary base sequences (*e.g.*, hybridization under "medium stringency" conditions may occur between homologs with about 50-70% identity). Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"High stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 0.1X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

"Medium stringency conditions" when used in reference to nucleic acid hybridization comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.5% SDS, 5X Denhardt's reagent and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising

1.0X SSPE, 1.0% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

"Low stringency conditions" comprise conditions equivalent to binding or hybridization at 42°C in a solution consisting of 5X SSPE (43.8 g/l NaCl, 6.9 g/l NaH₂PO₄•H₂O and 1.85 g/l EDTA, pH adjusted to 7.4 with NaOH), 0.1% SDS, 5X Denhardt's reagent [50X Denhardt's contains per 500 ml: 5 g Ficoll (Type 400, Pharmacia), 5 g BSA (Fraction V; Sigma)] and 100 µg/ml denatured salmon sperm DNA followed by washing in a solution comprising 5X SSPE, 0.1% SDS at 42°C when a probe of about 500 nucleotides in length is employed.

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA sequence given in a sequence listing or may comprise a complete gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (*i.e.*, a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local

homology algorithm of Smith and Waterman [Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981)] by the homology alignment algorithm of Needleman and Wunsch [Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970)], by the search for similarity method of Pearson and Lipman [Pearson and Lipman, *Proc. Natl. Acad. Sci. (U.S.A.)* 85:2444 (1988)], by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (*i.e.*, resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (*i.e.*, on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (*e.g.*, A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (*i.e.*, the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length sequences of the compositions claimed in the present invention (*e.g.*, Nod2)

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (*e.g.*, 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion as compared to the native protein, but where the remaining amino acid sequence is identical to the corresponding positions in the amino acid sequence deduced from a full-length cDNA sequence. Fragments typically are at least 4 amino acids long, preferably at least 20 amino acids long, usually at least 50 amino acids long or longer, and span the portion of the polypeptide required for intermolecular binding of the compositions(claimed in the present invention) with its various ligands and/or substrates.

The term "polymorphic locus" is a locus present in a population which shows variation between members of the population (*i.e.*, the most common allele has a frequency of less than 0.95). In contrast, a "monomorphic locus" is a genetic locus at little or no variations seen between members of the population (generally taken to be a locus at which the most common allele exceeds a frequency of 0.95 in the gene pool of the population).

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (*i.e.*, replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (*i.e.*, synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q β replicase, MDV-1 RNA is the specific template for the replicase (D.L. Kacian *et al.*, Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (M. Chamberlin *et al.*, Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (D.Y. Wu and R. B. Wallace, Genomics 4:560 [1989]). Finally, *Taq* and *Pfu* polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization

with the target sequences and not hybridization with non-target sequences (H.A. Erlich (ed.), *PCR Technology*, Stockton Press [1989]).

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids that may be amplified by any amplification method. It is contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target" (defined below). In contrast, "background template" is used in reference to nucleic acid other than sample template that may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, (*i.e.*, in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (*i.e.*, a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, that is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or

double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (*e.g.*, ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

As used herein, the term "target," when used in reference to the polymerase chain reaction, refers to the region of nucleic acid bounded by the primers used for polymerase chain reaction. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K.B. Mullis U.S. Patent Nos. 4,683,195, 4,683,202, and 4,965,188, hereby incorporated by reference, that describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing, and polymerase extension can be repeated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the

method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

5 With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (*e.g.*, hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to
10 genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process itself are, themselves, efficient templates for subsequent PCR amplifications.

As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms
15 encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the term "amplification reagents" refers to those reagents
20 (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template, and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the terms "restriction endonucleases" and "restriction enzymes"
25 refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques.

As used herein, the term "antisense" is used in reference to RNA sequences that are complementary to a specific RNA sequence (*e.g.*, mRNA). Included within this definition are antisense RNA ("asRNA") molecules involved in gene regulation by bacteria. Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter that permits the synthesis of a coding strand. Once introduced into an embryo, this transcribed strand combines with natural mRNA produced by the embryo to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (*i.e.*, "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (*i.e.*, "positive") strand.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids are nucleic acids such as DNA and RNA found in the state they exist in nature. For example, a given DNA sequence (*e.g.*, a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, isolated nucleic acid encoding Nod2 includes, by way of example, such nucleic acid in cells ordinarily expressing Nod2 where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or

coding strand (*i.e.*, the oligonucleotide or polynucleotide may single-stranded), but may contain both the sense and anti-sense strands (*i.e.*, the oligonucleotide or polynucleotide may be double-stranded).

As used herein, a "portion of a chromosome" refers to a discrete section of the chromosome. Chromosomes are divided into sites or sections by cytogeneticists as follows: the short (relative to the centromere) arm of a chromosome is termed the "p" arm; the long arm is termed the "q" arm. Each arm is then divided into 2 regions termed region 1 and region 2 (region 1 is closest to the centromere). Each region is further divided into bands. The bands may be further divided into sub-bands. For example, the 11p15.5 portion of human chromosome 11 is the portion located on chromosome 11 (11) on the short arm (p) in the first region (1) in the 5th band (5) in sub-band 5 (.5). A portion of a chromosome may be "altered;" for instance the entire portion may be absent due to a deletion or may be rearranged (*e.g.*, inversions, translocations, expanded or contracted due to changes in repeat regions). In the case of a deletion, an attempt to hybridize (*i.e.*, specifically bind) a probe homologous to a particular portion of a chromosome could result in a negative result (*i.e.*, the probe could not bind to the sample containing genetic material suspected of containing the missing portion of the chromosome). Thus, hybridization of a probe homologous to a particular portion of a chromosome may be used to detect alterations in a portion of a chromosome.

The term "sequences associated with a chromosome" means preparations of chromosomes (*e.g.*, spreads of metaphase chromosomes), nucleic acid extracted from a sample containing chromosomal DNA (*e.g.*, preparations of genomic DNA); the RNA that is produced by transcription of genes located on a chromosome (*e.g.*, hnRNA and mRNA), and cDNA copies of the RNA transcribed from the DNA located on a chromosome. Sequences associated with a chromosome may be detected by numerous techniques including probing of Southern and Northern blots and *in situ* hybridization to RNA, DNA, or metaphase chromosomes with probes containing sequences homologous to the nucleic acids in the above listed preparations.

As used herein the term "portion" when in reference to a nucleotide sequence (as in "a portion of a given nucleotide sequence") refers to fragments of that sequence. The fragments may range in size from four nucleotides to the entire nucleotide sequence minus one nucleotide (10 nucleotides, 20, 30, 40, 50, 100, 200, etc.).

As used herein the term "coding region" when used in reference to structural gene refers to the nucleotide sequences that encode the amino acids found in the nascent polypeptide as a result of translation of a mRNA molecule. The coding region is bounded, in eukaryotes, on the 5' side by the nucleotide triplet "ATG" that encodes the initiator methionine and on the 3' side by one of the three triplets which specify stop codons (*i.e.*, TAA, TAG, TGA).

As used herein, the term "purified" or "to purify" refers to the removal of contaminants from a sample. For example, Nod2 antibodies are purified by removal of contaminating non-immunoglobulin proteins; they are also purified by the removal of immunoglobulin that does not bind Nod2. The removal of non-immunoglobulin proteins and/or the removal of immunoglobulins that do not bind Nod2 results in an increase in the percent of Nod2-reactive immunoglobulins in the sample. In another example, recombinant Nod2 polypeptides are expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant Nod2 polypeptides is thereby increased in the sample.

The term "recombinant DNA molecule" as used herein refers to a DNA molecule that is comprised of segments of DNA joined together by means of molecular biological techniques.

The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule that is expressed from a recombinant DNA molecule.

The term "native protein" as used herein to indicate that a protein does not contain amino acid residues encoded by vector sequences; that is the native protein contains only those amino acids found in the protein as it occurs in nature. A native protein may be produced by recombinant means or may be isolated from a naturally occurring source.

As used herein the term "portion" when in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four consecutive amino acid residues to the entire amino acid sequence minus one amino acid.

5 The term "Southern blot," refers to the analysis of DNA on agarose or acrylamide gels to fractionate the DNA according to size followed by transfer of the DNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized DNA is then probed with a labeled probe to detect DNA species complementary to the probe used. The DNA may be cleaved with restriction enzymes
10 prior to electrophoresis. Following electrophoresis, the DNA may be partially depurinated and denatured prior to or during transfer to the solid support. Southern blots are a standard tool of molecular biologists (J. Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, NY, pp 9.31-9.58 [1989]).

15 The term "Northern blot," as used herein refers to the analysis of RNA by electrophoresis of RNA on agarose gels to fractionate the RNA according to size followed by transfer of the RNA from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized RNA is then probed with a labeled probe to detect RNA species complementary to the probe used. Northern blots are a standard tool of molecular biologists (J. Sambrook, *et al.*, *supra*, pp 7.39-7.52 [1989]).

20 The term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an antigen
25 of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabelled antibodies.

30 The term "antigenic determinant" as used herein refers to that portion of an antigen that makes contact with a particular antibody (*i.e.*, an epitope). When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies that bind specifically to a given

region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody.

5 The term "transgene" as used herein refers to a foreign gene that is placed into an organism by introducing the foreign gene into newly fertilized eggs or early embryos. The term "foreign gene" refers to any nucleic acid (*e.g.*, gene sequence) that is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not
10 reside in the same location as does the naturally-occurring gene. The term "autologous gene" is intended to encompass variants (*e.g.*, polymorphisms or mutants) of the naturally occurring gene. The term transgene thus encompasses the replacement of the naturally occurring gene with a variant form of the gene.

 As used herein, the term "vector" is used in reference to nucleic acid molecules
15 that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector."

 The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular
20 host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

 As used herein, the term "host cell" refers to any eukaryotic or prokaryotic cell
25 (*e.g.*, bacterial cells such as *E. coli*, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*. For example, host cells may be located in a transgenic animal.

 The terms "overexpression" and "overexpressing" and grammatical equivalents, are used in reference to levels of mRNA to indicate a level of expression

approximately 3-fold higher than that typically observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to those skilled in the art including, but not limited to Northern blot analysis (*See*, Example 10, for a protocol for performing Northern blot analysis).

Appropriate controls are included on the Northern blot to control for differences in the amount of RNA loaded from each tissue analyzed (*e.g.*, the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of normalizing or standardizing the RAD50 mRNA-specific signal observed on Northern blots). The amount of mRNA present in the band corresponding in size to the correctly spliced Nod2 transgene RNA is quantified; other minor species of RNA which hybridize to the transgene probe are not considered in the quantification of the expression of the transgenic mRNA.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell that has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells that have taken up foreign DNA but have failed to integrate this DNA.

The term "calcium phosphate co-precipitation" refers to a technique for the introduction of nucleic acids into a cell. The uptake of nucleic acids by cells is

enhanced when the nucleic acid is presented as a calcium phosphate-nucleic acid co-precipitate. The original technique of Graham and van der Eb (Graham and van der Eb, Virol., 52:456 [1973]), has been modified by several groups to optimize conditions for particular types of cells. The art is well aware of these numerous modifications.

A "composition comprising a given polynucleotide sequence" as used herein refers broadly to any composition containing the given polynucleotide sequence. The composition may comprise an aqueous solution. Compositions comprising polynucleotide sequences encoding Nod2 (*e.g.*, SEQ ID NO:1) or fragments thereof may be employed as hybridization probes. In this case, the Nod2 encoding polynucleotide sequences are typically employed in an aqueous solution containing salts (*e.g.*, NaCl), detergents (*e.g.*, SDS), and other components (*e.g.*, Denhardt's solution, dry milk, salmon sperm DNA, etc.).

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that can be used to treat or prevent a disease, illness, sickness, or disorder of bodily function, or otherwise alter the physiological or cellular status of a sample. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (*e.g.*, through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

The term "sample" as used herein is used in its broadest sense. A sample suspected of containing a human chromosome or sequences associated with a human chromosome may comprise a cell, chromosomes isolated from a cell (*e.g.*, a spread of metaphase chromosomes), genomic DNA (in solution or bound to a solid support such as for Southern blot analysis), RNA (in solution or bound to a solid support such as for Northern blot analysis), cDNA (in solution or bound to a solid support) and the like. A sample suspected of containing a protein may comprise a cell, a portion of a tissue, an extract containing one or more proteins and the like.

As used herein, the term "response," when used in reference to an assay, refers to the generation of a detectable signal (*e.g.*, accumulation of reporter protein, increase in ion concentration, accumulation of a detectable chemical product).

As used herein, the term "membrane receptor protein" refers to membrane spanning proteins that bind a ligand (*e.g.*, a hormone or neurotransmitter). As is known in the art, protein phosphorylation is a common regulatory mechanism used by cells to selectively modify proteins carrying regulatory signals from outside the cell to the nucleus. The proteins that execute these biochemical modifications are a group of enzymes known as protein kinases. They may further be defined by the substrate residue that they target for phosphorylation. One group of protein kinases are the tyrosine kinases (TKs) which selectively phosphorylate a target protein on its tyrosine residues. Some tyrosine kinases are membrane-bound receptors (RTKs), and, upon activation by a ligand, can autophosphorylate as well as modify substrates. The initiation of sequential phosphorylation by ligand stimulation is a paradigm that underlies the action of such effectors as, for example, epidermal growth factor (EGF), insulin, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF). The receptors for these ligands are tyrosine kinases and provide the interface between the binding of a ligand (hormone, growth factor) to a target cell and the transmission of a signal into the cell by the activation of one or more biochemical pathways. Ligand binding to a receptor tyrosine kinase activates its intrinsic enzymatic activity. Tyrosine kinases can also be cytoplasmic, non-receptor-type enzymes and act as a downstream component of a signal transduction pathway.

As used herein, the term "signal transduction protein" refers to a proteins that are activated or otherwise effected by ligand binding to a membrane receptor protein or some other stimulus. Examples of signal transduction protein include adenyl cyclase, phospholipase C, and G-proteins. Many membrane receptor proteins are coupled to G-proteins (*i.e.*, G-protein coupled receptors (GPCRs); for a review, *see* Neer, 1995, *Cell* 80:249-257 [1995]). Typically, GPCRs contain seven transmembrane domains. Putative GPCRs can be identified on the basis of sequence homology to known GPCRs.

GPCRs mediate signal transduction across a cell membrane upon the binding of a ligand to an extracellular portion of a GPCR. The intracellular portion of a GPCR interacts with a G-protein to modulate signal transduction from outside to inside a cell. A GPCR is therefore said to be "coupled" to a G-protein. G-proteins are composed of three polypeptide subunits: an α subunit, which binds and hydrolyses GTP, and a dimeric $\beta\gamma$ subunit. In the basal, inactive state, the G-protein exists as a heterotrimer of the α and $\beta\gamma$ subunits. When the G-protein is inactive, guanosine diphosphate (GDP) is associated with the α subunit of the G-protein. When a GPCR is bound and activated by a ligand, the GPCR binds to the G-protein heterotrimer and decreases the affinity of the $G\alpha$ subunit for GDP. In its active state, the G subunit exchanges GDP for guanine triphosphate (GTP) and active $G\alpha$ subunit disassociates from both the receptor and the dimeric $\beta\gamma$ subunit. The disassociated, active $G\alpha$ subunit transduces signals to effectors that are "downstream" in the G-protein signalling pathway within the cell. Eventually, the G-protein's endogenous GTPase activity returns active G subunit to its inactive state, in which it is associated with GDP and the dimeric $\beta\gamma$ subunit.

Numerous members of the heterotrimeric G-protein family have been cloned, including more than 20 genes encoding various $G\alpha$ subunits. The various G subunits have been categorized into four families, on the basis of amino acid sequences and functional homology. These four families are termed $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$. Functionally, these four families differ with respect to the intracellular signaling pathways that they activate and the GPCR to which they couple.

For example, certain GPCRs normally couple with $G\alpha_s$ and, through $G\alpha_s$, these GPCRs stimulate adenylyl cyclase activity. Other GPCRs normally couple with $GG\alpha_q$, and through $GG\alpha_q$, these GPCRs can activate phospholipase C (PLC), such as the β isoform of phospholipase C (*i.e.*, PLC β , Sternweis and Smrcka, Trends in Biochem. Sci. 17:502-506 [1992]).

As used herein, the term "nucleic acid binding protein" refers to proteins that bind to nucleic acid, and in particular to proteins that cause increased (*i.e.*, activators or transcription factors) or decreased (*i.e.*, inhibitors) transcription from a gene.

As used herein, the term “ion channel protein” refers to proteins that control the ingress or egress of ions across cell membranes. Examples of ion channel proteins include, but are not limited to, the Na⁺-K⁺ ATPase pump, the Ca²⁺ pump, and the K⁺ leak channel.

As used herein, the term “protein kinase” refers to proteins that catalyze the addition of a phosphate group from a nucleoside triphosphate to an amino acid side chain in a protein. Kinases comprise the largest known enzyme superfamily and vary widely in their target proteins. Kinases may be categorized as protein tyrosine kinases (PTKs), which phosphorylate tyrosine residues, and protein serine/threonine kinases (STKs), which phosphorylate serine and/or threonine residues. Some kinases have dual specificity for both serine/threonine and tyrosine residues. Almost all kinases contain a conserved 250-300 amino acid catalytic domain. This domain can be further divided into 11 subdomains. N-terminal subdomains I-IV fold into a two-lobed structure which binds and orients the ATP donor molecule, and subdomain V spans the two lobes. C-terminal subdomains VI-XI bind the protein substrate and transfer the gamma phosphate from ATP to the hydroxyl group of a serine, threonine, or tyrosine residue. Each of the 11 subdomains contains specific catalytic residues or amino acid motifs characteristic of that subdomain. For example, subdomain I contains an 8-amino acid glycine-rich ATP binding consensus motif, subdomain II contains a critical lysine residue required for maximal catalytic activity, and subdomains VI through IX comprise the highly conserved catalytic core. STKs and PTKs also contain distinct sequence motifs in subdomains VI and VIII which may confer hydroxyamino acid specificity. Some STKs and PTKs possess structural characteristics of both families. In addition, kinases may also be classified by additional amino acid sequences, generally between 5 and 100 residues, which either flank or occur within the kinase domain.

Non-transmembrane PTKs form signaling complexes with the cytosolic domains of plasma membrane receptors. Receptors that signal through non-transmembrane PTKs include cytokine, hormone, and antigen-specific lymphocytic receptors. Many PTKs were first identified as oncogene products in cancer cells in

which PTK activation was no longer subject to normal cellular controls. In fact, about one third of the known oncogenes encode PTKs. Furthermore, cellular transformation (oncogenesis) is often accompanied by increased tyrosine phosphorylation activity (*See, e.g., Carbonneau, H. and Tonks, Annu. Rev. Cell Biol. 8:463-93 [1992]*). Regulation of PTK activity may therefore be an important strategy in controlling some types of cancer.

As used herein, the term "protein phosphatase" refers to proteins that remove a phosphate group from a protein. Protein phosphatases are generally divided into two groups, receptor and non-receptor type proteins. Most receptor-type protein tyrosine phosphatases contain two conserved catalytic domains, each of which encompasses a segment of 240 amino acid residues. (*See, e.g., Saito et al., Cell Growth and Diff. 2:59-65 [1991]*). Receptor protein tyrosine phosphatases can be subclassified further based upon the amino acid sequence diversity of their extracellular domains. (*See, e.g., Krueger et al., Proc. Natl. Acad. Sci. USA 89:7417-7421 [1992]*).

As used herein, the term "reporter gene" refers to a gene encoding a protein that may be assayed. Examples of reporter genes include, but are not limited to, luciferase (*See, e.g., deWet et al., Mol. Cell. Biol. 7:725 [1987]* and U.S. Pat Nos., 6,074,859; 5,976,796; 5,674,713; and 5,618,682; all of which are incorporated herein by reference), green fluorescent protein (*e.g., GenBank Accession Number U43284*; a number of GFP variants are commercially available from CLONTECH Laboratories, Palo Alto, CA), chloramphenicol acetyltransferase, β -galactosidase, alkaline phosphatase, and horse radish peroxidase.

As used herein, the term "purified" refers to molecules, either nucleic or amino acid sequences, that are removed from their natural environment, isolated or separated. An "isolated nucleic acid sequence" is therefore a purified nucleic acid sequence. "Substantially purified" molecules are at least 60% free, preferably at least 75% free, and more preferably at least 90% free from other components with which they are naturally associated.

As used herein, the terms "computer memory" and "computer memory device" refer to any storage media readable by a computer processor. Examples of computer memory include, but are not limited to, RAM, ROM, computer chips, digital video disc (DVDs), compact discs (CDs), hard disk drives (HDD), and magnetic tape.

As used herein, the term "computer readable medium" refers to any device or system for storing and providing information (e.g., data and instructions) to a computer processor. Examples of computer readable media include, but are not limited to, DVDs, CDs, hard disk drives, magnetic tape and servers for streaming media over networks.

As used herein, the terms "processor" and "central processing unit" or "CPU" are used interchangeably and refer to a device that is able to read a program from a computer memory (e.g., ROM or other computer memory) and perform a set of steps according to the program.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to intracellular signalling molecules, in particular the Nod2 protein and nucleic acids encoding the Nod2 protein. The present invention encompasses both native and recombinant wild-type forms of Nod2, as well as mutant and variant forms, some of which possess altered characteristics relative to the wild-type Nod2. The present invention also relates to methods of using Nod2, including altered expression in transgenic organisms and expression in prokaryotes and cell culture systems. The present invention also encompasses methods for screening for drugs that inhibit or potentiate Nod2 action. The present invention also relates to methods for screening for susceptibility to intestinal bowel disease and Crohn's disease.

I. Nod2 Polynucleotides

As described above, a new family of proteins that activate NF- κ B have been discovered. This family was identified by screening public databases for nucleic acid sequences having homology to Nod1. Accordingly, the present invention provides

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nucleic acids encoding Nod2 genes, homologs, and variants (e.g., mutations and
polymorphisms (e.g., SEQ ID NOs: 1 and 33). In some embodiments, the present
invention provide polynucleotide sequences that are capable of hybridizing to SEQ ID
NOs:1 and 33 under conditions of low to high stringency as long as the polynucleotide
5 sequence capable of hybridizing encodes a protein that retains a biological activity of
the naturally occurring Nod2. In some embodiments, the protein that retains a
biological activity of naturally occurring Nod2 is 70% homologous to wild-type Nod2,
preferably 80% homologous to wild-type Nod2, more preferably 90% homologous to
wild-type Nod2, and most preferably 95% homologous to wild-type Nod2. In
10 preferred embodiments, hybridization conditions are based on the melting temperature
(T_m) of the nucleic acid binding complex and confer a defined "stringency" as
explained above (See e.g., Wahl, *et al.*, Meth. Enzymol., 152:399-407 [1987],
incorporated herein by reference).

15 In other embodiments of the present invention, alleles of Nod2 are provided.
In preferred embodiments, alleles result from a polymorphism or mutation, (*i.e.*, a
change in the nucleic acid sequence) and generally produce altered mRNAs or
polypeptides whose structure or function may or may not be altered. Any given gene
may have none, one or many allelic forms. Common mutational changes which give
rise to alleles are generally ascribed to deletions, additions or substitutions of nucleic
20 acids. Each of these types of changes may occur alone, or in combination with the
others, and at the rate of one or more times in a given sequence. Examples of the
alleles of the present invention include those encoded by (SEQ ID NOs:1 and 33;
wild-type and Crohn's disease mutant alleles, respectively).

25 In some embodiments of the present invention, the nucleic acids encode two
CARD domains corresponding (e.g., nucleic acid sequences encoding the peptides SEQ
ID NOs: 5 and 6). In other embodiments, the nucleic acids encode at least one
domain selected from the group consisting of an NBD domain (e.g., SEQ ID NO:7),
an LRR domain (e.g., SEQ ID NOs: 8-17), and P-loop and Mg^{2+} binding domains
(SEQ ID NO:18-19)

In still other embodiments of the present invention, the nucleotide sequences of the present invention may be engineered in order to alter an Nod2 coding sequence for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the gene product. For example, mutations may be introduced using techniques that are well known in the art (*e.g.*, site-directed mutagenesis to insert new restriction sites, to alter glycosylation patterns, to change codon preference, etc.).

In some embodiments of the present invention, the polynucleotide sequence of Nod2 may be extended utilizing the nucleotide sequences (*e.g.*, SEQ ID NOS:1 and 33) in various methods known in the art to detect upstream sequences such as promoters and regulatory elements. For example, it is contemplated that restriction-site polymerase chain reaction (PCR) will find use in the present invention. This is a direct method which uses universal primers to retrieve unknown sequence adjacent to a known locus (Gobinda *et al.*, PCR Methods Applic., 2:318-22 [1993]). First, genomic DNA is amplified in the presence of primer to a linker sequence and a primer specific to the known region. The amplified sequences are then subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

In another embodiment, inverse PCR can be used to amplify or extend sequences using divergent primers based on a known region (Triglia *et al.*, Nucleic Acids Res., 16:8186 [1988]). The primers may be designed using Oligo 4.0 (National Biosciences Inc, Plymouth Minn.), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72°C. The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template. In still other embodiments, walking PCR is utilized. Walking PCR is a method for targeted gene walking that permits retrieval of unknown sequence (Parker *et al.*, Nucleic Acids

Res., 19:3055-60 [1991]). The PROMOTERFINDER kit (Clontech) uses PCR, nested primers and special libraries to "walk in" genomic DNA. This process avoids the need to screen libraries and is useful in finding intron/exon junctions.

Preferred libraries for screening for full length cDNAs include mammalian libraries that have been size-selected to include larger cDNAs. Also, random primed libraries are preferred, in that they will contain more sequences which contain the 5' and upstream gene regions. A randomly primed library may be particularly useful in case where an oligo d(T) library does not yield full-length cDNA. Genomic mammalian libraries are useful for obtaining introns and extending 5' sequence.

In other embodiments of the present invention, variants of the disclosed Nod2 sequences are provided. In preferred embodiments, variants result from polymorphisms or mutations, (*i.e.*, a change in the nucleic acid sequence) and generally produce altered mRNAs or polypeptides whose structure or function may or may not be altered. Any given gene may have none, one, or many variant forms. Common mutational changes that give rise to variants are generally ascribed to deletions, additions or substitutions of nucleic acids. Each of these types of changes may occur alone, or in combination with the others, and at the rate of one or more times in a given sequence.

It is contemplated that it is possible to modify the structure of a peptide having a function (*e.g.*, Nod2 function) for such purposes as increasing binding affinity of the Nod2 for RICK. Such modified peptides are considered functional equivalents of peptides having an activity of Nod2 as defined herein. A modified peptide can be produced in which the nucleotide sequence encoding the polypeptide has been altered, such as by substitution, deletion, or addition. In particularly preferred embodiments, these modifications do not significantly reduce the synthetic activity of the modified Nod2. In other words, construct "X" can be evaluated in order to determine whether it is a member of the genus of modified or variant Nod2's of the present invention as defined functionally, rather than structurally. In preferred embodiments, the activity of a variant Nod2 is evaluated by the methods described in Example 4. Accordingly, in some embodiments, the present invention provides nucleic acids encoding a Nod2 that

activates NF- κ B (*e.g.*, activates an inflammatory response). In preferred embodiments, the activity of a Nod2 variant is evaluated by transfecting HEK293T cells with and expression construct encoding the variant Nod2. In particularly preferred
 5 regions that are responsive to NF- κ B. In other embodiments, the Nod2 variant may be capable of binding a protein (*e.g.*, RICK) but not activating NF- κ B. These variants can be screened for by the immunoprecipitation methods described in Example 6.

Moreover, as described above, variant forms of Nod2 are also contemplated as being equivalent to those peptides and DNA molecules that are set forth in more detail
 10 herein. For example, it is contemplated that isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (*i.e.*, conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Accordingly, some embodiments of the present invention provide
 15 variants of Nod2 disclosed herein containing conservative replacements. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine); (3) nonpolar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine,
 20 tryptophan); and (4) uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine). Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic (aspartate, glutamate); (2) basic (lysine, arginine, histidine), (3) aliphatic (glycine, alanine, valine, leucine, isoleucine, serine, threonine), with serine
 25 and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic (phenylalanine, tyrosine, tryptophan); (5) amide (asparagine, glutamine); and (6) sulfur-containing (cysteine and methionine) (*e.g.*, Stryer ed., *Biochemistry*, pg. 17-21, 2nd ed, WH Freeman and Co., 1981). Whether a change in the amino acid sequence of a peptide results in a functional homolog can be readily determined by assessing the

ability of the variant peptide to function in a fashion similar to the wild-type protein. Peptides having more than one replacement can readily be tested in the same manner.

More rarely, a variant includes "nonconservative" changes (*e.g.*, replacement of a glycine with a tryptophan). Analogous minor variations can also include amino acid deletions or insertions, or both. Guidance in determining which amino acid residues can be substituted, inserted, or deleted without abolishing biological activity can be found using computer programs (*e.g.*, LASERGENE software, DNASTAR Inc., Madison, Wis.).

As described in more detail below, variants may be produced by methods such as directed evolution or other techniques for producing combinatorial libraries of variants, described in more detail below. In still other embodiments of the present invention, the nucleotide sequences of the present invention may be engineered in order to alter a Nod2 coding sequence including, but not limited to, alterations that modify the cloning, processing, localization, secretion, and/or expression of the gene product. For example, mutations may be introduced using techniques that are well known in the art (*e.g.*, site-directed mutagenesis to insert new restriction sites, alter glycosylation patterns, or change codon preference, etc.).

II. Nod2 Polypeptides

In other embodiments, the present invention provides Nod2 polynucleotide sequences that encode Nod2 polypeptide sequences. Nod2 polypeptides (*e.g.*, SEQ ID NOs:2-3) are described in Figures 13 and 14. Other embodiments of the present invention provide fragments, fusion proteins or functional equivalents of these Nod2 proteins. In still other embodiment of the present invention, nucleic acid sequences corresponding to Nod2 variants, homologs, and mutants may be used to generate recombinant DNA molecules that direct the expression of the Nod2 variants, homologs and mutants in appropriate host cells. In some embodiments of the present invention, the polypeptide may be a naturally purified product, in other embodiments it may be a product of chemical synthetic procedures, and in still other embodiments it may be

produced by recombinant techniques using a prokaryotic or eukaryotic host (*e.g.*, by bacterial, yeast, higher plant, insect and mammalian cells in culture). In some embodiments, depending upon the host employed in a recombinant production procedure, the polypeptide of the present invention may be glycosylated or may be non-glycosylated. In other embodiments, the polypeptides of the invention may also include an initial methionine amino acid residue.

In one embodiment of the present invention, due to the inherent degeneracy of the genetic code, DNA sequences other than the polynucleotide sequences of SEQ ID NO:1 which encode substantially the same or a functionally equivalent amino acid sequence, may be used to clone and express Nod2. In general, such polynucleotide sequences hybridize to SEQ ID NO:1 under conditions of high to medium stringency as described above. As will be understood by those of skill in the art, it may be advantageous to produce Nod2-encoding nucleotide sequences possessing non-naturally occurring codons. Therefore, in some preferred embodiments, codons preferred by a particular prokaryotic or eukaryotic host (Murray *et al.*, Nucl. Acids Res., 17 [1989]) are selected, for example, to increase the rate of Nod2 expression or to produce recombinant RNA transcripts having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

1. Vectors for Production of Nod2

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. In some embodiments of the present invention, vectors include, but are not limited to, chromosomal, nonchromosomal and synthetic DNA sequences (*e.g.*, derivatives of SV40, bacterial plasmids, phage DNA; baculovirus, yeast plasmids, vectors derived from combinations of plasmids and phage DNA, and viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies). It is contemplated that any vector may be used as long as it is replicable and viable in the host.

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In particular, some embodiments of the present invention provide recombinant constructs comprising one or more of the sequences as broadly described above (*e.g.*, SEQ ID NOS:1 and 33). In some embodiments of the present invention, the constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In still other embodiments, the heterologous structural sequence (*e.g.*, SEQ ID NO:1) is assembled in appropriate phase with translation initiation and termination sequences. In preferred embodiments of the present invention, the appropriate DNA sequence is inserted into the vector using any of a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art.

Large numbers of suitable vectors are known to those of skill in the art, and are commercially available. Such vectors include, but are not limited to, the following vectors: 1) Bacterial -- pQE70, pQE60, pQE-9 (Qiagen), pBS, pD10, phagescript, psiX174, pbluescript SK, pBSKS, pNH8A, pNH16a, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia); and 2) Eukaryotic -- pWLNEO, pSV2CAT, pOG44, PXT1, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia). Any other plasmid or vector may be used as long as they are replicable and viable in the host. In some preferred embodiments of the present invention, mammalian expression vectors comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. In other embodiments, DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required non-transcribed genetic elements.

In certain embodiments of the present invention, the DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. Promoters useful in the present invention include, but are not limited to, the LTR or SV40 promoter, the *E. coli lac* or *trp*, the phage lambda P_L and P_R, T3 and T7 promoters, and the cytomegalovirus (CMV)

immediate early, herpes simplex virus (HSV) thymidine kinase, and mouse metallothionein-I promoters and other promoters known to control expression of gene in prokaryotic or eukaryotic cells or their viruses. In other embodiments of the present invention, recombinant expression vectors include origins of replication and selectable markers permitting transformation of the host cell (*e.g.*, dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or tetracycline or ampicillin resistance in *E. coli*).

In some embodiments of the present invention, transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes is increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act on a promoter to increase its transcription. Enhancers useful in the present invention include, but are not limited to, the SV40 enhancer on the late side of the replication origin bp 100 to 270, a cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

In other embodiments, the expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. In still other embodiments of the present invention, the vector may also include appropriate sequences for amplifying expression.

2. Host Cells for Production of Nod2

In a further embodiment, the present invention provides host cells containing the above-described constructs. In some embodiments of the present invention, the host cell is a higher eukaryotic cell (*e.g.*, a mammalian or insect cell). In other embodiments of the present invention, the host cell is a lower eukaryotic cell (*e.g.*, a yeast cell). In still other embodiments of the present invention, the host cell can be a prokaryotic cell (*e.g.*, a bacterial cell). Specific examples of host cells include, but are not limited to, *Escherichia coli*, *Salmonella typhimurium*, *Bacillus subtilis*, and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, as

well as *Saccharomyces cerivisiae*, *Schizosaccharomyces pombe*, *Drosophila* S2 cells, *Spodoptera* Sf9 cells, Chinese hamster ovary (CHO) cells, COS-7 lines of monkey kidney fibroblasts, (Gluzman, Cell 23:175 [1981]), C127, 3T3, 293, 293T, HeLa and BHK cell lines.

5 The constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. In some embodiments, introduction of the construct into the host cell can be accomplished by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (*See e.g.*, Davis *et al.*, Basic Methods in Molecular Biology, [1986]). Alternatively, in
10 some embodiments of the present invention, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

 Proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of
15 the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, N.Y., (1989).

 In some embodiments of the present invention, following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (*e.g.*, temperature shift or chemical
20 induction) and cells are cultured for an additional period. In other embodiments of the present invention, cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. In still other embodiments of the present invention, microbial cells employed in
25 expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

3. Purification of Nod2

The present invention also provides methods for recovering and purifying Nod2 from recombinant cell cultures including, but not limited to, ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. In other embodiments of the present invention, protein refolding steps can be used as necessary, in completing configuration of the mature protein. In still other embodiments of the present invention, high performance liquid chromatography (HPLC) can be employed for final purification steps.

The present invention further provides polynucleotides having the coding sequence (*e.g.*, SEQ ID NOs: 1 and 33) fused in frame to a marker sequence which allows for purification of the polypeptide of the present invention. A non-limiting example of a marker sequence is a hexahistidine tag which may be supplied by a vector, preferably a pQE-9 vector, which provides for purification of the polypeptide fused to the marker in the case of a bacterial host, or, for example, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host (*e.g.*, COS-7 cells) is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, Cell, 37:767 [1984]).

4. Truncation Mutants of Nod2

In addition, the present invention provides fragments of Nod2 (*i.e.*, truncation mutants, *e.g.*, SEQ ID NO:3). In some embodiments of the present invention, when expression of a portion of the Nod2 protein is desired, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat *et al.*, J. Bacteriol., 169:751-757 [1987]) and *Salmonella typhimurium* and its *in vitro* activity has been

demonstrated on recombinant proteins (Miller *et al.*, Proc. Natl. Acad. Sci. USA 84:2718-1722 [1990]). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing such recombinant polypeptides in a host which produces MAP (*e.g.*, *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP.

5. Fusion Proteins Containing Nod2

The present invention also provides fusion proteins incorporating all or part of Nod2. Accordingly, in some embodiments of the present invention, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is contemplated that this type of expression system will find use under conditions where it is desirable to produce an immunogenic fragment of a Nod2 protein. In some embodiments of the present invention, the VP6 capsid protein of rotavirus is used as an immunologic carrier protein for portions of the Nod2 polypeptide, either in the monomeric form or in the form of a viral particle. In other embodiments of the present invention, the nucleic acid sequences corresponding to the portion of Nod2 against which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of Nod2 as part of the virion. It has been demonstrated with the use of immunogenic fusion proteins utilizing the hepatitis B surface antigen fusion proteins that recombinant hepatitis B virions can be utilized in this role as well. Similarly, in other embodiments of the present invention, chimeric constructs coding for fusion proteins containing a portion of Nod2 and the poliovirus capsid protein are created to enhance immunogenicity of the set of polypeptide antigens (*See e.g.*, EP Publication No. 025949; and Evans *et al.*, Nature 339:385 [1989]; Huang *et al.*, J. Virol., 62:3855 [1988]; and Schlienger *et al.*, J. Virol., 66:2 [1992]).

In still other embodiments of the present invention, the multiple antigen peptide system for peptide-based immunization can be utilized. In this system, a desired portion of Nod2 is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (*see e.g.*, Posnett *et al.*, J. Biol. Chem., 263:1719 [1988]; and Nardelli *et al.*, J. Immunol., 148:914 [1992]). In other
5 embodiments of the present invention, antigenic determinants of the Nod2 proteins can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins, such as
10 the Nod2 protein of the present invention. Accordingly, in some embodiments of the present invention, Nod2 can be generated as a glutathione-S-transferase (*i.e.*, GST fusion protein). It is contemplated that such GST fusion proteins will enable easy purification of Nod2, such as by the use of glutathione-derivatized matrices (*See e.g.*, Ausabel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons,
15 NY [1991]). In another embodiment of the present invention, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of Nod2, can allow purification of the expressed Nod2 fusion protein by affinity chromatography using a Ni²⁺ metal resin. In still another embodiment of the present invention, the purification leader sequence can
20 then be subsequently removed by treatment with enterokinase (*See e.g.*, Hochuli *et al.*, J. Chromatogr., 411:177 [1987]; and Janknecht *et al.*, Proc. Natl. Acad. Sci. USA 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in
25 accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment of the present invention, the fusion gene can be synthesized by conventional techniques including

automated DNA synthesizers. Alternatively, in other embodiments of the present invention, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (See e.g., Current Protocols in Molecular Biology, *supra*).

6. Variants of Nod2

Still other embodiments of the present invention provide mutant or variant forms of Nod2 (*i.e.*, muteins). It is possible to modify the structure of a peptide having an activity of Nod2 for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (*e.g.*, *ex vivo* shelf life, and/or resistance to proteolytic degradation *in vivo*). Such modified peptides are considered functional equivalents of peptides having an activity of the subject Nod2 proteins as defined herein. A modified peptide can be produced in which the amino acid sequence has been altered, such as by amino acid substitution, deletion, or addition.

Moreover, as described above, variant forms (*e.g.*, mutants or polymorphic sequences) of the subject Nod2 proteins are also contemplated as being equivalent to those peptides and DNA molecules that are set forth in more detail. For example, as described above, the present invention encompasses mutant and variant proteins that contain conservative or non-conservative amino acid substitutions.

This invention further contemplates a method of generating sets of combinatorial mutants of the present Nod2 proteins, as well as truncation mutants, and is especially useful for identifying potential variant sequences (*i.e.*, mutants or polymorphic sequences) that are functional in binding to NF- κ B or proteins in the NF- κ B signalling pathway and signalling an inflammatory response. The purpose of screening such combinatorial libraries is to generate, for example, novel Nod2 variants which can act as either agonists or antagonists, or alternatively, possess novel activities all together.

Therefore, in some embodiments of the present invention, Nod2 variants are engineered by the present method to provide more efficient activation of NF- κ B (*i.e.*, generating an inflammatory response). In other embodiments of the present invention, combinatorially-derived homologs are generated which have a selective potency relative to a naturally occurring Nod2. Such proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols.

Still other embodiments of the present invention provide Nod2 variants which have intracellular half-lives dramatically different than the corresponding wild-type protein. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivate Nod2. Such variants, and the genes which encode them, can be utilized to alter the location of Nod2 expression by modulating the half-life of the protein. For instance, a short half-life can give rise to more transient Nod2 biological effects and, when part of an inducible expression system, can allow tighter control of Nod2 levels within the cell. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

In still other embodiments of the present invention, Nod2 variants are generated by the combinatorial approach to act as antagonists, in that they are able to interfere with the ability of the corresponding wild-type protein to regulate cell function.

In some embodiments of the combinatorial mutagenesis approach of the present invention, the amino acid sequences for a population of Nod2 variants or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, Nod2 homologs from one or more species, or Nod2 variants from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences.

In a preferred embodiment of the present invention, the combinatorial Nod2 library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential Nod2 protein sequences. For example, a mixture of synthetic oligonucleotides can be enzymatically ligated into

gene sequences such that the degenerate set of potential Nod2 sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of Nod2 sequences therein.

There are many ways by which the library of potential Nod2 homologs and variants can be generated from a degenerate oligonucleotide sequence. In some embodiments, chemical synthesis of a degenerate gene sequence is carried out in an automatic DNA synthesizer, and the synthetic genes are ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential Nod2 sequences. The synthesis of degenerate oligonucleotides is well known in the art (*See e.g.*, Narang, Tetrahedron Lett., 39:3 9 [1983]; Itakura *et al.*, Recombinant DNA, in Walton (ed.), *Proceedings of the 3rd Cleveland Symposium on Macromolecules*, Elsevier, Amsterdam, pp 273-289 [1981]; Itakura *et al.*, Annu. Rev. Biochem., 53:323 [1984]; Itakura *et al.*, Science 198:1056 [1984]; Ike *et al.*, Nucl. Acid Res., 11:477 [1983]). Such techniques have been employed in the directed evolution of other proteins (*See e.g.*, Scott *et al.*, Science 249:386-390 [1980]; Roberts *et al.*, Proc. Natl. Acad. Sci. USA 89:2429-2433 [1992]; Devlin *et al.*, Science 249: 404-406 [1990]; Cwirla *et al.*, Proc. Natl. Acad. Sci. USA 87: 6378-6382 [1990]; as well as U.S. Pat. Nos. 5,223,409, 5,198,346, and 5,096,815, each of which is incorporated herein by reference).

It is contemplated that the Nod2 nucleic acids (e.g., SEQ ID NO: 1, and fragments and variants thereof) can be utilized as starting nucleic acids for directed evolution. These techniques can be utilized to develop Nod2 variants having desirable properties such as increased or decreased binding affinity for RICK.

In some embodiments, artificial evolution is performed by random mutagenesis (e.g., by utilizing error-prone PCR to introduce random mutations into a given coding sequence). This method requires that the frequency of mutation be finely tuned. As a general rule, beneficial mutations are rare, while deleterious mutations are common. This is because the combination of a deleterious mutation and a beneficial mutation

often results in an inactive enzyme. The ideal number of base substitutions for targeted gene is usually between 1.5 and 5 (Moore and Arnold, *Nat. Biotech.*, 14, 458-67 [1996]; Leung *et al.*, *Technique*, 1:11-15 [1989]; Eckert and Kunkel, *PCR Methods Appl.*, 1:17-24 [1991]; Caldwell and Joyce, *PCR Methods Appl.*, 2:28-33 (1992); and Zhao and Arnold, *Nuc. Acids. Res.*, 25:1307-08 [1997]). After mutagenesis, the resulting clones are selected for desirable activity (*e.g.*, screened for Nod2 activity). Successive rounds of mutagenesis and selection are often necessary to develop enzymes with desirable properties. It should be noted that only the useful mutations are carried over to the next round of mutagenesis.

In other embodiments of the present invention, the polynucleotides of the present invention are used in gene shuffling or sexual PCR procedures (*e.g.*, Smith, *Nature*, 370:324-25 [1994]; U.S. Pat. Nos. 5,837,458; 5,830,721; 5,811,238; 5,733,731; all of which are herein incorporated by reference). Gene shuffling involves random fragmentation of several mutant DNAs followed by their reassembly by PCR into full length molecules. Examples of various gene shuffling procedures include, but are not limited to, assembly following DNase treatment, the staggered extension process (STEP), and random priming in vitro recombination. In the DNase mediated method, DNA segments isolated from a pool of positive mutants are cleaved into random fragments with DNaseI and subjected to multiple rounds of PCR with no added primer. The lengths of random fragments approach that of the uncleaved segment as the PCR cycles proceed, resulting in mutations in present in different clones becoming mixed and accumulating in some of the resulting sequences. Multiple cycles of selection and shuffling have led to the functional enhancement of several enzymes (Stemmer, *Nature*, 370:398-91 [1994]; Stemmer, *Proc. Natl. Acad. Sci. USA*, 91, 10747-51 [1994]; Cramer *et al.*, *Nat. Biotech.*, 14:315-19 [1996]; Zhang *et al.*, *Proc. Natl. Acad. Sci. USA*, 94:4504-09 [1997]; and Cramer *et al.*, *Nat. Biotech.*, 15:436-38 [1997]). Variants produced by directed evolution can be screened for Nod2 activity by the methods described in Examples 4-8.

5 A wide range of techniques are known in the art for screening gene products of
combinatorial libraries made by point mutations, and for screening cDNA libraries for
gene products having a certain property. Such techniques will be generally adaptable
for rapid screening of the gene libraries generated by the combinatorial mutagenesis or
recombination of Nod2 homologs. The most widely used techniques for screening
large gene libraries typically comprises cloning the gene library into replicable
expression vectors, transforming appropriate cells with the resulting library of vectors,
and expressing the combinatorial genes under conditions in which detection of a
desired activity facilitates relatively easy isolation of the vector encoding the gene
10 whose product was detected.

7. Chemical Synthesis of Nod2

15 In an alternate embodiment of the invention, the coding sequence of Nod2 is
synthesized, whole or in part, using chemical methods well known in the art (*See e.g.*,
Caruthers *et al.*, Nucl. Acids Res. Symp. Ser., 7:215-233 [1980]; Crea and Horn, Nucl.
Acids Res., 9:2331 [1980]; Matteucci and Caruthers, Tetrahedron Lett., 21:719 [1980];
and Chow and Kempe, Nucl. Acids Res., 9:2807-2817 [1981]). In other embodiments
of the present invention, the protein itself is produced using chemical methods to
synthesize either an entire Nod2 amino acid sequence or a portion thereof. For
example, peptides can be synthesized by solid phase techniques, cleaved from the
20 resin, and purified by preparative high performance liquid chromatography (*See e.g.*,
Creighton, *Proteins Structures And Molecular Principles*, W H Freeman and Co, New
York N.Y. [1983]). In other embodiments of the present invention, the composition of
the synthetic peptides is confirmed by amino acid analysis or sequencing (*See e.g.*,
Creighton, *supra*).

25 Direct peptide synthesis can be performed using various solid-phase techniques
(Roberge *et al.*, Science 269:202-204 [1995]) and automated synthesis may be
achieved, for example, using ABI 431A Peptide Synthesizer (Perkin Elmer) in
accordance with the instructions provided by the manufacturer. Additionally, the

amino acid sequence of Nod2, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with other sequences to produce a variant polypeptide.

III. Detection of Nod2 Alleles

5 In some embodiments, the present invention includes alleles of Nod2 that increase a patient's susceptibility to Crohn's disease (*e.g.*, including, but not limited to, SEQ ID NOs: 33 and 3). Analysis of naturally occurring human Nod2 alleles revealed that patients with increased susceptibility to Crohn's disease have a mutant Nod2 allele that, for example, contains an additional cytosine residue. The additional cytosine
10 residue causes a frameshift mutation resulting in the generation of a stop codon that causes deletion of much of the LRR domain. The resulting protein functions similarly to deletion mutant Δ LRR (See Fig 4). This deletion mutant exhibits a high degree of NF- κ B activation in the absence of other signalling stimuli. However, the present invention is not limited to the mutation described in SEQ ID NOs: 3 and 33. Any
15 mutation that results in the undesired phenotype (*e.g.*, a high degree of NF- κ B activation in the absence of other signalling stimuli) is within the scope of the present invention. Assays for determining if a given polypeptide has such activities are provided in Examples 4 and 5.

The present invention is not limited to a particular mechanism of action.
20 Indeed, an understanding of the mechanism of action is not necessary to practice the present invention. Nevertheless, it is contemplated that the signaling system of which Nod2 is a component recognizes bacterial and viral pathogens and initiates a response to kill the pathogen or infected cell (*i.e.*, cell signalling that activates a transcription factor, that in turn, activates an inflammatory response). It is contemplated that in
25 Crohn's disease the activation of the signalling pathway occurs in the absence of pathogen stimulation because of the presence of the truncated form of Nod2. This leads to the inflammation associated with Crohn's disease.

Accordingly, the present invention provides methods for determining whether a patient has an increased susceptibility to inflammatory bowel disease or Crohn's

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disease by determining whether the individual has a variant Nod2 gene. In other embodiments, the present invention provides methods for providing a prognosis of increased risk for Crohn's disease to an individual based on the presence or absence of one or more variant alleles of Nod2. In preferred embodiments, the variation causes a truncation of the LRR domain. In other preferred embodiments, the variation results in increased activation of NF- κ B and consequent inflammatory response. In particularly preferred embodiments, the variation is single nucleotide polymorphism caused by an insertion of a cytosine residue.

A number of methods are available for analysis of variant (*e.g.*, mutant or polymorphic) nucleic acid sequences. Assays for detections variants (*e.g.*, polymorphisms or mutations) fall into several categories, including, but not limited to direct sequencing assays, fragment polymorphism assays, hybridization assays, and computer based data analysis. Protocols and commercially available kits or services for performing multiple variations of these assays are available. In some embodiments, assays are performed in combination or in hybrid (*e.g.*, different reagents or technologies from several assays are combined to yield one assay). The following assays are useful in the present invention.

1. Direct sequencing Assays

In some embodiments of the present invention, variant sequences are detected using a direct sequencing technique. In these assays, DNA samples are first isolated from a subject using any suitable method. In some embodiments, the region of interest is cloned into a suitable vector and amplified by growth in a host cell (*e.g.*, a bacteria). In other embodiments, DNA in the region of interest is amplified using PCR.

Following amplification, DNA in the region of interest (*e.g.*, the region containing the SNP or mutation of interest) is sequenced using any suitable method, including but not limited to manual sequencing using radioactive marker nucleotides, or automated sequencing. The results of the sequencing are displayed using any

suitable method. The sequence is examined and the presence or absence of a given SNP or mutation is determined.

2. PCR Assay

In some embodiments of the present invention, variant sequences are detected using a PCR-based assay. In some embodiments, the PCR assay comprises the use of oligonucleotide primers that hybridize only to the variant or wild type allele of Nod2 (e.g., to the region of polymorphism or mutation). Both sets of primers are used to amplify a sample of DNA. If only the mutant primers result in a PCR product, then the patient has the mutant Nod2 allele. If only the wild-type primers result in a PCR product, then the patient has the wild type allele of Nod2.

3. Fragment Length Polymorphism Assays

In some embodiments of the present invention, variant sequence are detected using a fragment length polymorphism assay. In a fragment length polymorphism assay, a unique DNA banding pattern based on cleaving the DNA at a series of positions is generated using an enzyme (e.g., a restriction enzyme or a CLEAVASE I [Third Wave Technologies, Madison, WI] enzyme). DNA fragments from a sample containing a SNP or a mutation will have a different banding pattern than wild type.

a. RFLP Assay

In some embodiments of the present invention, variant sequences are detected using a restriction fragment length polymorphism assay (RFLP). The region of interest is first isolated using PCR. The PCR products are then cleaved with restriction enzymes known to give a unique length fragment for a given polymorphism. The restriction-enzyme digested PCR products are separated by agarose gel electrophoresis and visualized by ethidium bromide staining. The length of the fragments is compared to molecular weight markers and fragments generated from wild-type and mutant controls.

b. CFLP Assay

In other embodiments, variant sequences are detected using a CLEAVASE fragment length polymorphism assay (CFLP; Third Wave Technologies, Madison, WI; *See e.g.*, U.S. Patent Nos. 5,843,654; 5,843,669; 5,719,208; and 5,888,780; each of which is herein incorporated by reference). This assay is based on the observation that when single strands of DNA fold on themselves, they assume higher order structures that are highly individual to the precise sequence of the DNA molecule. These secondary structures involve partially duplexed regions of DNA such that single stranded regions are juxtaposed with double stranded DNA hairpins. The CLEAVASE I enzyme, is a structure-specific, thermostable nuclease that recognizes and cleaves the junctions between these single-stranded and double-stranded regions.

The region of interest is first isolated, for example, using PCR. Then, DNA strands are separated by heating. Next, the reactions are cooled to allow intrastrand secondary structure to form. The PCR products are then treated with the CLEAVASE I enzyme to generate a series of fragments that are unique to a given SNP or mutation. The CLEAVASE enzyme treated PCR products are separated and detected (*e.g.*, by agarose gel electrophoresis) and visualized (*e.g.*, by ethidium bromide staining). The length of the fragments is compared to molecular weight markers and fragments generated from wild-type and mutant controls.

4. Hybridization Assays

In preferred embodiments of the present invention, variant sequences are detected a hybridization assay. In a hybridization assay, the presence of absence of a given SNP or mutation is determined based on the ability of the DNA from the sample to hybridize to a complementary DNA molecule (*e.g.*, a oligonucleotide probe). A variety of hybridization assays using a variety of technologies for hybridization and detection are available. A description of a selection of assays is provided below.

a. Direct Detection of Hybridization

In some embodiments, hybridization of a probe to the sequence of interest (*e.g.*, a SNP or mutation) is detected directly by visualizing a bound probe (*e.g.*, a Northern or Southern assay; *See e.g.*, Ausabel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY [1991]). In a these assays, genomic DNA (Southern) or RNA (Northern) is isolated from a subject. The DNA or RNA is then cleaved with a series of restriction enzymes that cleave infrequently in the genome and not near any of the markers being assayed. The DNA or RNA is then separated (*e.g.*, on an agarose gel) and transferred to a membrane. A labelled (*e.g.*, by incorporating a radionucleotide) probe or probes specific for the SNP or mutation being detected is allowed to contact the membrane under a condition or low, medium, or high stringency conditions. Unbound probe is removed and the presence of binding is detected by visualizing the labelled probe.

b. Detection of Hybridization Using "DNA Chip" Assays

In some embodiments of the present invention, variant sequences are detected using a DNA chip hybridization assay. In this assay, a series of oligonucleotide probes are affixed to a solid support. The oligonucleotide probes are designed to be unique to a given SNP or mutation. The DNA sample of interest is contacted with the DNA "chip" and hybridization is detected.

In some embodiments, the DNA chip assay is a GeneChip (Affymetrix, Santa Clara, CA; *See e.g.*, U.S. Patent Nos. 6,045,996; 5,925,525; and 5,858,659; each of which is herein incorporated by reference) assay. The GeneChip technology uses miniaturized, high-density arrays of oligonucleotide probes affixed to a "chip." Probe arrays are manufactured by Affymetrix's light-directed chemical synthesis process, which combines solid-phase chemical synthesis with photolithographic fabrication techniques employed in the semiconductor industry. Using a series of photolithographic masks to define chip exposure sites, followed by specific chemical synthesis steps, the process constructs high-density arrays of oligonucleotides, with

each probe in a predefined position in the array. Multiple probe arrays are synthesized simultaneously on a large glass wafer. The wafers are then diced, and individual probe arrays are packaged in injection-molded plastic cartridges, which protect them from the environment and serve as chambers for hybridization.

5 The nucleic acid to be analyzed is isolated, amplified by PCR, and labeled with a fluorescent reporter group. The labeled DNA is then incubated with the array using a fluidics station. The array is then inserted into the scanner, where patterns of hybridization are detected. The hybridization data are collected as light emitted from the fluorescent reporter groups already incorporated into the target, which is bound to the probe array. Probes that perfectly match the target generally produce stronger signals than those that have mismatches. Since the sequence and position of each probe on the array are known, by complementarity, the identity of the target nucleic acid applied to the probe array can be determined.

10 In other embodiments, a DNA microchip containing electronically captured probes (Nanogen, San Diego, CA) is utilized (*See e.g.*, U.S. Patent Nos. 6,017,696; 6,068,818; and 6,051,380; each of which are herein incorporated by reference). Through the use of microelectronics, Nanogen's technology enables the active movement and concentration of charged molecules to and from designated test sites on its semiconductor microchip. DNA capture probes unique to a given SNP or mutation are electronically placed at, or "addressed" to, specific sites on the microchip. Since DNA has a strong negative charge, it can be electronically moved to an area of positive charge.

15 First, a test site or a row of test sites on the microchip is electronically activated with a positive charge. Next, a solution containing the DNA probes is introduced onto the microchip. The negatively charged probes rapidly move to the positively charged sites, where they concentrate and are chemically bound to a site on the microchip. The microchip is then washed and another solution of distinct DNA probes is added until the array of specifically bound DNA probes is complete.

20 A test sample is then analyzed for the presence of target DNA molecules by determining which of the DNA capture probes hybridize, with complementary DNA in

the test sample (*e.g.*, a PCR amplified gene of interest). An electronic charge is also used to move and concentrate target molecules to one or more test sites on the microchip. The electronic concentration of sample DNA at each test site promotes rapid hybridization of sample DNA with complementary capture probes (hybridization may occur in minutes). To remove any unbound or nonspecifically bound DNA from each site, the polarity or charge of the site is reversed to negative, thereby forcing any unbound or nonspecifically bound DNA back into solution away from the capture probes. A laser-based fluorescence scanner is used to detect binding,

In still further embodiments, an array technology based upon the segregation of fluids on a flat surface (chip) by differences in surface tension (ProtoGene, Palo Alto, CA) is utilized (*See e.g.*, U.S. Patent Nos. 6,001,311; 5,985,551; and 5,474,796; each of which is herein incorporated by reference). Protogene's technology is based on the fact that fluids can be segregated on a flat surface by differences in surface tension that have been imparted by chemical coatings. Once so segregated, oligonucleotide probes are synthesized directly on the chip by ink-jet printing of reagents. The array with its reaction sites defined by surface tension is mounted on a X/Y translation stage under a set of four piezoelectric nozzles, one for each of the four standard DNA bases. The translation stage moves along each of the rows of the array and the appropriate reagent is delivered to each of the reaction site. For example, the A amidite is delivered only to the sites where amidite A is to be coupled during that synthesis step and so on. Common reagents and washes are delivered by flooding the entire surface and then removing them by spinning.

DNA probes unique for the SNP or mutation of interest are affixed to the chip using Protogene's technology. The chip is then contacted with the PCR-amplified genes of interest. Following hybridization, unbound DNA is removed and hybridization is detected using any suitable method (*e.g.*, by fluorescence de-quenching of an incorporated fluorescent group).

In yet other embodiments, a "bead array" is used for the detection of polymorphisms (Illumina, San Diego, CA; *See e.g.*, PCT Publications WO 99/67641 and WO 00/39587, each of which is herein incorporated by reference). Illumina uses

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a BEAD ARRAY technology that combines fiber optic bundles and beads that self-assemble into an array. Each fiber optic bundle contains thousands to millions of individual fibers depending on the diameter of the bundle. The beads are coated with an oligonucleotide specific for the detection of a given SNP or mutation. Batches of beads are combined to form a pool specific to the array. To perform an assay, the BEAD ARRAY is contacted with a prepared subject sample (e.g., DNA). Hybridization is detected using any suitable method.

c. Enzymatic Detection of Hybridization

In some embodiments of the present invention, genomic profiles are generated using a assay that detects hybridization by enzymatic cleavage of specific structures (INVADER assay, Third Wave Technologies; *See e.g.*, U.S. Patent Nos. 5,846,717; 6,090,543; 6,001,567; 5,985,557; and 5,994,069; each of which is herein incorporated by reference). The INVADER assay detects specific DNA and RNA sequences by using structure-specific enzymes to cleave a complex formed by the hybridization of overlapping oligonucleotide probes. Elevated temperature and an excess of one of the probes enable multiple probes to be cleaved for each target sequence present without temperature cycling. These cleaved probes then direct cleavage of a second labeled probe. The secondary probe oligonucleotide can be 5'-end labeled with fluorescein that is quenched by an internal dye. Upon cleavage, the de-quenched fluorescein labeled product may be detected using a standard fluorescence plate reader.

The INVADER assay detects specific mutations and SNPs in unamplified genomic DNA. The isolated DNA sample is contacted with the first probe specific either for a SNP/mutation or wild type sequence and allowed to hybridize. Then a secondary probe, specific to the first probe, and containing the fluorescein label, is hybridized and the enzyme is added. Binding is detected by using a fluorescent plate reader and comparing the signal of the test sample to known positive and negative controls.

In some embodiments, hybridization of a bound probe is detected using a TaqMan assay (PE Biosystems, Foster City, CA; *See e.g.*, U.S. Patent Nos. 5,962,233

and 5,538,848, each of which is herein incorporated by reference). The assay is performed during a PCR reaction. The TaqMan assay exploits the 5'-3' exonuclease activity of the AMPLITAQ GOLD DNA polymerase. A probe, specific for a given allele or mutation, is included in the PCR reaction. The probe consists of an oligonucleotide with a 5'-reporter dye (*e.g.*, a fluorescent dye) and a 3'-quencher dye. During PCR, if the probe is bound to its target, the 5'-3' nucleolytic activity of the AMPLITAQ GOLD polymerase cleaves the probe between the reporter and the quencher dye. The separation of the reporter dye from the quencher dye results in an increase of fluorescence. The signal accumulates with each cycle of PCR and can be monitored with a fluorimeter.

In still further embodiments, polymorphisms are detected using the SNP-IT primer extension assay (Orchid Biosciences, Princeton, NJ; *See e.g.*, U.S. Patent Nos. 5,952,174 and 5,919,626, each of which is herein incorporated by reference). In this assay, SNPs are identified by using a specially synthesized DNA primer and a DNA polymerase to selectively extend the DNA chain by one base at the suspected SNP location. DNA in the region of interest is amplified and denatured. Polymerase reactions are then performed using miniaturized systems called microfluidics. Detection is accomplished by adding a label to the nucleotide suspected of being at the SNP or mutation location. Incorporation of the label into the DNA can be detected by any suitable method (*e.g.*, if the nucleotide contains a biotin label, detection is via a fluorescently labelled antibody specific for biotin).

5. Mass Spectroscopy Assay

In some embodiments, a MassARRAY system (Sequenom, San Diego, CA.) is used to detect variant sequences (*See e.g.*, U.S. Patent Nos. 6,043,031; 5,777,324; and 5,605,798; each of which is herein incorporated by reference). DNA is isolated from blood samples using standard procedures. Next, specific DNA regions containing the mutation or SNP of interest, about 200 base pairs in length, are amplified by PCR. The amplified fragments are then attached by one strand to a solid surface and the non-immobilized strands are removed by standard denaturation and washing. The

remaining immobilized single strand then serves as a template for automated enzymatic reactions that produce genotype specific diagnostic products.

Very small quantities of the enzymatic products, typically five to ten nanoliters, are then transferred to a SpectroCHIP array for subsequent automated analysis with the SpectroREADER mass spectrometer. Each spot is preloaded with light absorbing crystals that form a matrix with the dispensed diagnostic product. The MassARRAY system uses MALDI-TOF (Matrix Assisted Laser Desorption Ionization - Time of Flight) mass spectrometry. In a process known as desorption, the matrix is hit with a pulse from a laser beam. Energy from the laser beam is transferred to the matrix and it is vaporized resulting in a small amount of the diagnostic product being expelled into a flight tube. As the diagnostic product is charged when an electrical field pulse is subsequently applied to the tube they are launched down the flight tube towards a detector. The time between application of the electrical field pulse and collision of the diagnostic product with the detector is referred to as the time of flight. This is a very precise measure of the product's molecular weight, as a molecule's mass correlates directly with time of flight with smaller molecules flying faster than larger molecules. The entire assay is completed in less than one thousandth of a second, enabling samples to be analyzed in a total of 3-5 second including repetitive data collection. The SpectroTYPER software then calculates, records, compares and reports the genotypes at the rate of three seconds per sample.

6. Variant Analysis by Differential Antibody Binding

In other embodiments of the present invention, antibodies (See below for antibody production) are used to determine if an individual contains an allele encoding a variant Nod2 gene. In preferred embodiments, antibodies are utilized that discriminate between mutant (*i.e.*, truncated proteins); and wild-type proteins (SEQ ID NOs:2 and 3). In some particularly preferred embodiments, the antibodies are directed to the C-terminus of Nod2.

7. Kits for Analyzing Risk of Crohn's Disease

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The present invention also provides kits for determining whether an individual contains a wild-type or variant (*e.g.*, polymorphic or mutant) allele of Nod2. In some embodiments, the kits are useful determining whether the subject is at risk of developing Crohn's disease. The diagnostic kits are produced in a variety of ways. In some embodiments, the kits contain at least one reagent for specifically detecting a mutant Nod2 allele or protein. In preferred embodiments, the kits contains reagents for detecting a SNP caused by an insertion of a cytosine residue into the wild-type gene. In preferred embodiments, the reagent is a nucleic acid that hybridizes to nucleic acids containing the SNP and that does not bind to nucleic acids that do not contain the SNP. In other preferred embodiments, the reagents are primers for amplifying the region of DNA containing the SNP. In still other embodiments, the reagents are antibodies which preferentially bind either the wild-type or truncated Nod2 proteins. In some embodiments, the kit contains instructions for determining whether the subject is at risk for developing Crohn's disease. In preferred embodiments, the instructions specify that risk for developing Crohn's disease is determined by detecting the presence or absence of a mutant Nod2 allele in the subject, wherein subjects having an allele containing a cytosine insertion mutation have an increased risk of developing Crohn's disease. In some embodiments, the kits include ancillary reagents such as buffering agents, nucleic acid stabilizing reagents, protein stabilizing reagents, and signal producing systems (*e.g.*, florescence generating systems as Fret systems). The test kit may be packages in any suitable manner, typically with the elements in a single container or various containers as necessary along with a sheet of instructions for carrying out the test. in some embodiments, the kits also preferably include a positive control sample.

IV. Generation of Nod2 Antibodies

Antibodies can be generated to allow for the detection of Nod2 protein. The antibodies may be prepared using various immunogens. In one embodiment, the immunogen is a human Nod2 peptide to generate antibodies that recognize human

Nod2. Such antibodies include, but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and Fab expression libraries.

Various procedures known in the art may be used for the production of polyclonal antibodies directed against Nod2. For the production of antibody, various host animals can be immunized by injection with the peptide corresponding to the Nod2 epitope including but not limited to rabbits, mice, rats, sheep, goats, etc. In a preferred embodiment, the peptide is conjugated to an immunogenic carrier (e.g., diphtheria toxoid, bovine serum albumin (BSA), or keyhole limpet hemocyanin (KLH)). Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels (e.g., aluminum hydroxide), surface active substances (e.g., lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (Bacille Calmette-Guerin) and *Corynebacterium parvum*).

For preparation of monoclonal antibodies directed toward Nod2, it is contemplated that any technique that provides for the production of antibody molecules by continuous cell lines in culture will find use with the present invention (See e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). These include but are not limited to the hybridoma technique originally developed by Köhler and Milstein (Köhler and Milstein, *Nature* 256:495-497 [1975]), as well as the trioma technique, the human B-cell hybridoma technique (See e.g., Kozbor *et al.*, *Immunol. Tod.*, 4:72 [1983]), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96 [1985]).

In an additional embodiment of the invention, monoclonal antibodies are produced in germ-free animals utilizing technology such as that described in PCT/US90/02545). Furthermore, it is contemplated that human antibodies will be generated by human hybridomas (Cote *et al.*, *Proc. Natl. Acad. Sci. USA*

80:2026-2030 [1983]) or by transforming human B cells with EBV virus *in vitro* (Cole *et al.*, in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96 [1985]).

In addition, it is contemplated that techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; herein incorporated by reference) will find use in producing Nod2 specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse *et al.*, Science 246:1275-1281 [1989]) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity for Nod2.

It is contemplated that any technique suitable for producing antibody fragments will find use in generating antibody fragments that contain the idiotype (antigen binding region) of the antibody molecule. For example, such fragments include but are not limited to: F(ab')₂ fragment that can be produced by pepsin digestion of the antibody molecule; Fab' fragments that can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and Fab fragments that can be generated by treating the antibody molecule with papain and a reducing agent.

In the production of antibodies, it is contemplated that screening for the desired antibody will be accomplished by techniques known in the art (*e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (*e.g.*, using colloidal gold, enzyme or radioisotope labels, for example), Western blots, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays, etc.), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc.

In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the

art for detecting binding in an immunoassay and are within the scope of the present invention. (As is well known in the art, the immunogenic peptide should be provided free of the carrier molecule used in any immunization protocol. For example, if the peptide was conjugated to KLH, it may be conjugated to BSA, or used directly, in a screening assay.)

The foregoing antibodies can be used in methods known in the art relating to the localization and structure of Nod2 (*e.g.*, for Western blotting), measuring levels thereof in appropriate biological samples, etc. The antibodies can be used to detect Nod2 in a biological sample from an individual. The biological sample can be a biological fluid, such as, but not limited to, blood, serum, plasma, interstitial fluid, urine, cerebrospinal fluid, and the like, containing cells.

The biological samples can then be tested directly for the presence of human Nod2 using an appropriate strategy (*e.g.*, ELISA or radioimmunoassay) and format (*e.g.*, microwells, dipstick (*e.g.*, as described in International Patent Publication WO 93/03367), etc. Alternatively, proteins in the sample can be size separated (*e.g.*, by polyacrylamide gel electrophoresis (PAGE), in the presence or not of sodium dodecyl sulfate (SDS), and the presence of Nod2 detected by immunoblotting (Western blotting). Immunoblotting techniques are generally more effective with antibodies generated against a peptide corresponding to an epitope of a protein, and hence, are particularly suited to the present invention.

Another method uses antibodies as agents to alter signal transduction. Specific antibodies that bind to the binding domains of Nod2 or other proteins involved in intracellular signalling can be used to inhibit the interaction between the various proteins and their interaction with other ligands. Antibodies that bind to the complex can also be used therapeutically to inhibit interactions of the protein complex in the signal transduction pathways leading to the various physiological and cellular effects of NF- κ B. Such antibodies can also be used diagnostically to measure abnormal expression of Nod2, or the aberrant formation of protein complexes, which may be indicative of a disease state.

V. Gene Therapy Using Nod2

The present invention also provides methods and compositions suitable for gene therapy to alter Nod2 expression, production, or function. As described above, the present invention provides human Nod2 genes and provides methods of obtaining Nod2 genes from other species. Thus, the methods described below are generally applicable across many species. In some embodiments, it is contemplated that the gene therapy is performed by providing a subject with a wild-type allele of Nod2 (*i.e.*, an allele that does contain a cytosine insertion mutation). Subjects in need of such therapy are identified by the methods described above. As described above, Nod2 is primarily expressed in the monocytes. Accordingly, a preferred method of gene therapy is to ablate the subjects monocytes (*e.g.*, via radiation) and replace the defective monocytes with monocytes expressing wild-type Nod2 via a bone marrow transplant. In some embodiments, the subjects defective monocytes may be harvested prior to radiation treatment, transfected with a vector (described below) encoding wild-type monocytes, amplified through *in vitro* cultured, and reintroduced into the subject.

Viral vectors commonly used for *in vivo* or *ex vivo* targeting and therapy procedures are DNA-based vectors and retroviral vectors. Methods for constructing and using viral vectors are known in the art (*See e.g.*, Miller and Rosman, BioTech., 7:980-990 [1992]). Preferably, the viral vectors are replication defective, that is, they are unable to replicate autonomously in the target cell. In general, the genome of the replication defective viral vectors that are used within the scope of the present invention lack at least one region that is necessary for the replication of the virus in the infected cell. These regions can either be eliminated (in whole or in part), or be rendered non-functional by any technique known to a person skilled in the art. These techniques include the total removal, substitution (by other sequences, in particular by the inserted nucleic acid), partial deletion or addition of one or more bases to an essential (for replication) region. Such techniques may be performed *in vitro* (*i.e.*, on the isolated DNA) or *in situ*, using the techniques of genetic manipulation or by treatment with mutagenic agents.

Preferably, the replication defective virus retains the sequences of its genome that are necessary for encapsidating the viral particles. DNA viral vectors include an attenuated or defective DNA viruses, including, but not limited to, herpes simplex virus (HSV), papillomavirus, Epstein Barr virus (EBV), adenovirus, adeno-associated virus (AAV), and the like. Defective viruses, that entirely or almost entirely lack viral genes, are preferred, as defective virus is not infective after introduction into a cell. Use of defective viral vectors allows for administration to cells in a specific, localized area, without concern that the vector can infect other cells. Thus, a specific tissue can be specifically targeted. Examples of particular vectors include, but are not limited to, a defective herpes virus 1 (HSV1) vector (Kaplitt *et al.*, Mol. Cell. Neurosci., 2:320-330 [1991]), defective herpes virus vector lacking a glycoprotein L gene (*See e.g.*, Patent Publication RD 371005 A), or other defective herpes virus vectors (*See e.g.*, WO 94/21807; and WO 92/05263); an attenuated adenovirus vector, such as the vector described by Stratford-Perricaudet *et al.* (J. Clin. Invest., 90:626-630 [1992]; *See also*, La Salle *et al.*, Science 259:988-990 [1993]); and a defective adeno-associated virus vector (Samulski *et al.*, J. Virol., 61:3096-3101 [1987]; Samulski *et al.*, J. Virol., 63:3822-3828 [1989]; and Lebkowski *et al.*, Mol. Cell. Biol., 8:3988-3996 [1988]).

Preferably, for *in vivo* administration, an appropriate immunosuppressive treatment is employed in conjunction with the viral vector (*e.g.*, adenovirus vector), to avoid immuno-deactivation of the viral vector and transfected cells. For example, immunosuppressive cytokines, such as interleukin-12 (IL-12), interferon-gamma (IFN- γ), or anti-CD4 antibody, can be administered to block humoral or cellular immune responses to the viral vectors. In addition, it is advantageous to employ a viral vector that is engineered to express a minimal number of antigens.

In a preferred embodiment, the vector is an adenovirus vector. Adenoviruses are eukaryotic DNA viruses that can be modified to efficiently deliver a nucleic acid of the invention to a variety of cell types. Various serotypes of adenovirus exist. Of these serotypes, preference is given, within the scope of the present invention, to type

2 or type 5 human adenoviruses (Ad 2 or Ad 5), or adenoviruses of animal origin (*See e.g.*, WO94/26914). Those adenoviruses of animal origin that can be used within the scope of the present invention include adenoviruses of canine, bovine, murine (*e.g.*, Mav1, Beard *et al.*, Virol., 75-81 [1990]), ovine, porcine, avian, and simian (*e.g.*, SAV) origin. Preferably, the adenovirus of animal origin is a canine adenovirus, more preferably a CAV2 adenovirus (*e.g.* Manhattan or A26/61 strain (ATCC VR-800)).

Preferably, the replication defective adenoviral vectors of the invention comprise the ITRs, an encapsidation sequence and the nucleic acid of interest. Still more preferably, at least the E1 region of the adenoviral vector is non-functional. The deletion in the E1 region preferably extends from nucleotides 455 to 3329 in the sequence of the Ad5 adenovirus (*Pvu*II-*Bgl*II fragment) or 382 to 3446 (*Hinf*II-*Sau*3A fragment). Other regions may also be modified, in particular the E3 region (*e.g.*, WO95/02697), the E2 region (*e.g.*, WO94/28938), the E4 region (*e.g.*, WO94/28152, WO94/12649 and WO95/02697), or in any of the late genes L1-L5.

In a preferred embodiment, the adenoviral vector has a deletion in the E1 region (Ad 1.0). Examples of E1-deleted adenoviruses are disclosed in EP 185,573, the contents of which are incorporated herein by reference. In another preferred embodiment, the adenoviral vector has a deletion in the E1 and E4 regions (Ad 3.0). Examples of E1/E4-deleted adenoviruses are disclosed in WO95/02697 and WO96/22378. In still another preferred embodiment, the adenoviral vector has a deletion in the E1 region into which the E4 region and the nucleic acid sequence are inserted.

The replication defective recombinant adenoviruses according to the invention can be prepared by any technique known to the person skilled in the art (*See e.g.*, Levrero *et al.*, Gene 101:195 [1991]; EP 185 573; and Graham, EMBO J., 3:2917 [1984]). In particular, they can be prepared by homologous recombination between an adenovirus and a plasmid which carries, *inter alia*, the DNA sequence of interest. The homologous recombination is accomplished following co-transfection of the adenovirus and plasmid into an appropriate cell line. The cell line that is employed should

preferably (i) be transformable by the elements to be used, and (ii) contain the sequences that are able to complement the part of the genome of the replication defective adenovirus, preferably in integrated form in order to avoid the risks of recombination. Examples of cell lines that may be used are the human embryonic kidney cell line 293 (Graham *et al.*, J. Gen. Virol., 36:59 [1977]), which contains the left-hand portion of the genome of an Ad5 adenovirus (12%) integrated into its genome, and cell lines that are able to complement the E1 and E4 functions, as described in applications WO94/26914 and WO95/02697. Recombinant adenoviruses are recovered and purified using standard molecular biological techniques, that are well known to one of ordinary skill in the art.

The adeno-associated viruses (AAV) are DNA viruses of relatively small size that can integrate, in a stable and site-specific manner, into the genome of the cells that they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions that carry the encapsidation functions: the left-hand part of the genome, that contains the *rep* gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, that contains the *cap* gene encoding the capsid proteins of the virus.

The use of vectors derived from the AAVs for transferring genes *in vitro* and *in vivo* has been described (See *e.g.*, WO 91/18088; WO 93/09239; US Pat. No. 4,797,368; US Pat. No., 5,139,941; and EP 488 528, all of which are herein incorporated by reference). These publications describe various AAV-derived constructs in which the *rep* and/or *cap* genes are deleted and replaced by a gene of interest, and the use of these constructs for transferring the gene of interest *in vitro* (into cultured cells) or *in vivo* (directly into an organism). The replication defective

recombinant AAVs according to the invention can be prepared by co-transfecting a plasmid containing the nucleic acid sequence of interest flanked by two AAV inverted terminal repeat (ITR) regions, and a plasmid carrying the AAV encapsidation genes (*rep* and *cap* genes), into a cell line that is infected with a human helper virus (for example an adenovirus). The AAV recombinants that are produced are then purified by standard techniques.

In another embodiment, the gene can be introduced in a retroviral vector (e.g., as described in U.S. Pat. Nos. 5,399,346, 4,650,764, 4,980,289 and 5,124,263; all of which are herein incorporated by reference; Mann *et al.*, Cell 33:153 [1983]; Markowitz *et al.*, J. Virol., 62:1120 [1988]; PCT/US95/14575; EP 453242; EP178220; Bernstein *et al.* Genet. Eng., 7:235 [1985]; McCormick, BioTechnol., 3:689 [1985]; WO 95/07358; and Kuo *et al.*, Blood 82:845 [1993]). The retroviruses are integrating viruses that infect dividing cells. The retrovirus genome includes two LTRs, an encapsidation sequence and three coding regions (*gag*, *pol* and *env*). In recombinant retroviral vectors, the *gag*, *pol* and *env* genes are generally deleted, in whole or in part, and replaced with a heterologous nucleic acid sequence of interest. These vectors can be constructed from different types of retrovirus, such as, HIV, MoMuLV ("murine Moloney leukaemia virus" MSV ("murine Moloney sarcoma virus"), HaSV ("Harvey sarcoma virus"); SNV ("spleen necrosis virus"); RSV ("Rous sarcoma virus") and Friend virus. Defective retroviral vectors are also disclosed in WO95/02697.

In general, in order to construct recombinant retroviruses containing a nucleic acid sequence, a plasmid is constructed that contains the LTRs, the encapsidation sequence and the coding sequence. This construct is used to transfect a packaging cell line, which cell line is able to supply in trans the retroviral functions that are deficient in the plasmid. In general, the packaging cell lines are thus able to express the *gag*, *pol* and *env* genes. Such packaging cell lines have been described in the prior art, in particular the cell line PA317 (US Pat. No. 4,861,719, herein incorporated by reference), the PsiCRIP cell line (See, WO90/02806), and the GP+envAm-12 cell line (See, WO89/07150). In addition, the recombinant retroviral vectors can contain

modifications within the LTRs for suppressing transcriptional activity as well as extensive encapsidation sequences that may include a part of the *gag* gene (Bender *et al.*, J. Virol., 61:1639 [1987]). Recombinant retroviral vectors are purified by standard techniques known to those having ordinary skill in the art.

5 Alternatively, the vector can be introduced *in vivo* by lipofection. For the past decade, there has been increasing use of liposomes for encapsulation and transfection of nucleic acids *in vitro*. Synthetic cationic lipids designed to limit the difficulties and dangers encountered with liposome mediated transfection can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker (Felgner *et al.*, Proc. Natl. Acad. Sci. USA 84:7413-7417 [1987]; *See also*, Mackey, *et al.*, Proc. Natl. Acad. Sci. USA 85:8027-8031 [1988]; Ulmer *et al.*, Science 259:1745-1748 [1993]). The use of cationic lipids may promote encapsulation of negatively charged nucleic acids, and also promote fusion with negatively charged cell membranes (Felgner and Ringold, Science 337:387-388 [1989]). Particularly useful lipid compounds and compositions for transfer of nucleic acids are described in WO95/18863 and WO96/17823, and in U.S. Pat. No. 5,459,127, herein incorporated by reference.

Other molecules are also useful for facilitating transfection of a nucleic acid *in vivo*, such as a cationic oligopeptide (*e.g.*, WO95/21931), peptides derived from DNA binding proteins (*e.g.*, WO96/25508), or a cationic polymer (*e.g.*, WO95/21931).

20 It is also possible to introduce the vector *in vivo* as a naked DNA plasmid. Methods for formulating and administering naked DNA to mammalian muscle tissue are disclosed in U.S. Pat. Nos. 5,580,859 and 5,589,466, both of which are herein incorporated by reference.

DNA vectors for gene therapy can be introduced into the desired host cells by methods known in the art, including but not limited to transfection, electroporation, microinjection, transduction, cell fusion, DEAE dextran, calcium phosphate precipitation, use of a gene gun, or use of a DNA vector transporter (*See e.g.*, Wu *et al.*, J. Biol. Chem., 267:963-967 [1992]; Wu and Wu, J. Biol. Chem., 263:14621-14624 [1988]; and Williams *et al.*, Proc. Natl. Acad. Sci. USA

88:2726-2730 [1991])). Receptor-mediated DNA delivery approaches can also be used (Curiel *et al.*, Hum. Gene Ther., 3:147-154 [1992]; and Wu and Wu, J. Biol. Chem., 262:4429-4432 [1987])).

VI. Transgenic Animals Expressing Exogenous Nod2 Genes and Homologs, Mutants, and Variants Thereof

The present invention contemplates the generation of transgenic animals comprising an exogenous Nod2 gene or homologs, mutants, or variants thereof. In preferred embodiments, the transgenic animal displays an altered phenotype as compared to wild-type animals. In some embodiments, the altered phenotype is the overexpression of mRNA for a Nod2 gene as compared to wild-type levels of Nod2 expression. In other embodiments, the altered phenotype is the decreased expression of mRNA for an endogenous Nod2 gene as compared to wild-type levels of endogenous Nod2 expression. Methods for analyzing the presence or absence of such phenotypes include Northern blotting, mRNA protection assays, and RT-PCR. In other embodiments, the transgenic mice have a knock out mutation of the Nod2 gene. In still further embodiments, expression of a Nod2 variant gene (*e.g.*, SEQ ID NO:33 (the c insertion mutant) or mutants containing deletions of one or more LRR repeats). In preferred embodiments, the transgenic animals display a Crohn's disease phenotype.

The transgenic animals of the present invention find use in dietary and drug screens. In some embodiments, the transgenic animals (*e.g.*, animals displaying a Crohn's disease phenotype) are fed test or control diets and the response of the animals to the diets is evaluated. In other embodiments, test compounds (*e.g.*, a drug that is suspected of being useful to treat Crohn's disease) and control compounds (*e.g.*, a placebo) are administered to the transgenic animals and the control animals and the effects evaluated.

The transgenic animals can be generated via a variety of methods. In some embodiments, embryonal cells at various developmental stages are used to introduce transgenes for the production of transgenic animals. Different methods are used

depending on the stage of development of the embryonal cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible injection of 1-2 picoliters (pl) of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster *et al.*, Proc. Natl. Acad. Sci. USA 82:4438-4442 [1985]). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. U.S. Patent No. 4,873,191 describes a method for the micro-injection of zygotes; the disclosure of this patent is incorporated herein in its entirety.

In other embodiments, retroviral infection is used to introduce transgenes into a non-human animal. In some embodiments, the retroviral vector is utilized to transfect oocytes by injecting the retroviral vector into the perivitelline space of the oocyte (U.S. Pat. No. 6,080,912, incorporated herein by reference). In other embodiments, the developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Janenich, Proc. Natl. Acad. Sci. USA 73:1260-1264 [1976]). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan *et al.*, in *Manipulating the Mouse Embryo*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. [1986]). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (D. Jahner *et al.*, Proc. Natl. Acad. Sci. USA 82:6927-693 [1985]). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart, *et al.*, EMBO J., 6:383-388 [1987]). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (D. Jahner *et al.*, Nature 298:623-628 [1982]). Most of the founders will be mosaic for the transgene since incorporation occurs only in a

subset of cells which form the transgenic animal. Further, the founder may contain various retroviral insertions of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germline, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner *et al.*, supra [1982]). Additional means of using retroviruses or retroviral vectors to create transgenic animals known to the art involves the micro-injection of retroviral particles or mitomycin C-treated cells producing retrovirus into the perivitelline space of fertilized eggs or early embryos (PCT International Application WO 90/08832 [1990], and Haskell and Bowen, Mol. Reprod. Dev., 40:386 [1995]).

In other embodiments, the transgene is introduced into embryonic stem cells and the transfected stem cells are utilized to form an embryo. ES cells are obtained by culturing pre-implantation embryos *in vitro* under appropriate conditions (Evans *et al.*, Nature 292:154-156 [1981]; Bradley *et al.*, Nature 309:255-258 [1984]; Gossler *et al.*, Proc. Acad. Sci. USA 83:9065-9069 [1986]; and Robertson *et al.*, Nature 322:445-448 [1986]). Transgenes can be efficiently introduced into the ES cells by DNA transfection by a variety of methods known to the art including calcium phosphate coprecipitation, protoplast or spheroplast fusion, lipofection and DEAE-dextran-mediated transfection. Transgenes may also be introduced into ES cells by retrovirus-mediated transduction or by micro-injection. Such transfected ES cells can thereafter colonize an embryo following their introduction into the blastocoel of a blastocyst-stage embryo and contribute to the germ line of the resulting chimeric animal (for review, See, Jaenisch, Science 240:1468-1474 [1988]). Prior to the introduction of transfected ES cells into the blastocoel, the transfected ES cells may be subjected to various selection protocols to enrich for ES cells which have integrated the transgene assuming that the transgene provides a means for such selection. Alternatively, the polymerase chain reaction may be used to screen for ES cells which have integrated the transgene. This technique obviates the need for growth of the transfected ES cells under appropriate selective conditions prior to transfer into the blastocoel.

In still other embodiments, homologous recombination is utilized knock-out gene function or create deletion mutants (*e.g.*, mutants in which the LRRs of Nod2 are deleted). Methods for homologous recombination are described in U.S. Pat. No. 5,614,396, incorporated herein by reference.

VII. Transgenic Plants Expressing Exogenous Nod2 and Homologs, Mutants, and Variants Thereof

As described above, the plant Nod2 homologs share homology with a class of plant disease resistant R gene products. The present invention provides transgenic plants and methods for creating transgenic plants that have altered responses and or resistance to pathogens. In some embodiments, the transgenic plants express an exogenous Nod2 gene or homolog, mutant or variant thereof (*e.g.*, SEQ ID NOs: 1 and 33). In preferred embodiments, the transgenic plant displays an altered phenotype as compared to wild-type plants. In some embodiments, the altered phenotype is the overexpression of mRNA for a Nod2 gene as compared to wild-type levels of Nod2 expression. In other embodiments, the altered phenotype is the decreased expression of mRNA for an endogenous Nod2 gene as compared to wild-type levels of endogenous Nod2 expression. Methods for analyzing the presence or absence of such phenotypes include Northern blotting, mRNA protection assays, and RT-PCR. In still further embodiments, increased Nod2 gene expression in the transgenic plant confers increased resistance to pathogens. In some embodiments, the observed phenotype mimics the inflammatory response induced by Nod2 in animals. Transgenic plants expressing this phenotype may be screened by challenging plants with a pathogen and selecting plants that display resistance as compared to control, nontransgenic plants.

In some embodiments of the present invention, vectors are provided for the transfection of plant hosts to create transgenic plants. In general, these vectors comprise a Nod2 nucleic acid (*e.g.*, SEQ ID NOs:1 and 33) operably linked to a promoter and other regulatory sequences (*e.g.*, enhancers, polyadenylation signals, etc.) required for expression in a plant. The Nod2 nucleic acid can be oriented to produce sense or antisense transcripts, depending on the desired use. In some embodiments,

the promoter is a constitutive promoter (e.g., superpromoter or SD promoter). In other embodiments, the promoter is a seed specific promoter (e.g., phaseolin promoter [See e.g., U.S. Pat. No. 5,589,616, incorporated herein by reference], napin promoter [See e.g., U.S. Pat. No. 5,608,152, incorporated herein by reference], or acyl-CoA carrier protein promoter [See e.g., 5,767,363, incorporated herein by reference]).

In some preferred embodiments, the vector is adapted for use in an *Agrobacterium* mediated transfection process (See e.g., U.S. Pat. Nos. 5,981,839; 6,051,757; 5,981,840; 5,824,877; and 4,940,838; all of which are incorporated herein by reference). Construction of recombinant Ti and Ri plasmids in general follows methods typically used with the more common bacterial vectors, such as pBR322. Additional use can be made of accessory genetic elements sometimes found with the native plasmids and sometimes constructed from foreign sequences. These may include but are not limited to structural genes for antibiotic resistance as selection genes.

There are two systems of recombinant Ti and Ri plasmid vector systems now in use. The first system is called the "cointegrate" system. In this system, the shuttle vector containing the gene of interest is inserted by genetic recombination into a non-oncogenic Ti plasmid that contains both the cis-acting and trans-acting elements required for plant transformation as, for example, in the pMLJ1 shuttle vector and the non-oncogenic Ti plasmid pGV3850. The second system is called the "binary" system in which two plasmids are used; the gene of interest is inserted into a shuttle vector containing the cis-acting elements required for plant transformation. The other necessary functions are provided in trans by the non-oncogenic Ti plasmid as exemplified by the pBIN19 shuttle vector and the non-oncogenic Ti plasmid PAL4404. Some of these vectors are commercially available.

It may be desirable to target the nucleic acid sequence of interest to a particular locus on the plant genome. Site-directed integration of the nucleic acid sequence of interest into the plant cell genome may be achieved by, for example, homologous recombination using *Agrobacterium*-derived sequences. Generally, plant cells are

incubated with a strain of *Agrobacterium* which contains a targeting vector in which sequences that are homologous to a DNA sequence inside the target locus are flanked by *Agrobacterium* transfer-DNA (T-DNA) sequences, as previously described (U.S.

Pat. No. 5,501,967, the entire contents of which are herein incorporated by reference).

5 One of skill in the art knows that homologous recombination may be achieved using targeting vectors which contain sequences that are homologous to any part of the targeted plant gene, whether belonging to the regulatory elements of the gene, or the coding regions of the gene. Homologous recombination may be achieved at any region of a plant gene so long as the nucleic acid sequence of regions flanking the site
10 to be targeted is known.

The nucleic acids of the present invention may also be utilized to construct vectors derived from plant (+) RNA viruses (*e.g.*, brome mosaic virus, tobacco mosaic virus, alfalfa mosaic virus, cucumber mosaic virus, tomato mosaic virus, and combinations and hybrids thereof). Generally, the inserted Nod2 polynucleotide can
15 be expressed from these vectors as a fusion protein (*e.g.*, coat protein fusion protein) or from its own subgenomic promoter or other promoter. Methods for the construction and use of such viruses are described in U.S. Pat. Nos. 5,846,795; 5,500,360; 5,173,410; 5,965,794; 5,977,438; and 5,866,785, all of which are incorporated herein by reference.

20 Alternatively, vectors can be constructed for expression in hosts other than plants (*e.g.*, prokaryotic cells such as *E. coli*, yeast cells, *C. elegans*, and mammalian cell culture cells). In some embodiments of the present invention, vectors include, but are not limited to, chromosomal, nonchromosomal and synthetic DNA sequences (*e.g.*, derivatives of SV40, bacterial plasmids, phage DNA; baculovirus, yeast plasmids,
25 vectors derived from combinations of plasmids and phage DNA, and viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies). Large numbers of suitable vectors that are replicable and viable in the host are known to those of skill in the art, and are commercially available. Any other plasmid or vector may be used as long as they are replicable and viable in the host.

In some preferred embodiments of the present invention, bacterial expression vectors comprise an origin of replication, a suitable promoter and optionally an enhancer, and also any necessary ribosome binding sites, polyadenylation sites, transcriptional termination sequences, and 5' flanking nontranscribed sequences.

5 Promoters useful in the present invention include, but are not limited to, retroviral LTRs, SV40 promoter, CMV promoter, RSV promoter, *E. coli lac* or *trp* promoters, phage lambda P_L and P_R promoters, T3, SP6 and T7 promoters. In other embodiments of the present invention, recombinant expression vectors include origins of replication and selectable markers, (e.g., tetracycline or ampicillin resistance in *E.*

10 *coli*, or neomycin phosphotransferase gene for selection in eukaryotic cells).

The vectors described above can be utilized to express the Nod2 of the present invention in transgenic plants. A variety of methods are known for producing transgenic plants.

In some embodiments, *Agrobacterium* mediated transfection is utilized to create

15 transgenic plants. Since most dicotyledonous plant are natural hosts for *Agrobacterium*, almost every dicotyledonous plant may be transformed by *Agrobacterium in vitro*. Although monocotyledonous plants, and in particular, cereals and grasses, are not natural hosts to *Agrobacterium*, work to transform them using *Agrobacterium* has also been carried out (Hooykas-Van Slogteren *et al.*, Nature

20 311:763-764 [1984]). Plant genera that may be transformed by *Agrobacterium* include *Arabidopsis*, *Chrysanthemum*, *Dianthus*, *Gerbera*, *Euphorbia*, *Pelargonium*, *Ipomoea*, *Passiflora*, *Cyclamen*, *Malus*, *Prunus*, *Rosa*, *Rubus*, *Populus*, *Santalum*, *Allium*, *Lilium*, *Narcissus*, *Ananas*, *Arachis*, *Phaseolus* and *Pisum*.

For transformation with *Agrobacterium*, disarmed *Agrobacterium* cells are

25 transformed with recombinant Ti plasmids of *Agrobacterium tumefaciens* or Ri plasmids of *Agrobacterium rhizogenes* (such as those described in U.S. Patent No. 4,940,838, the entire contents of which are herein incorporated by reference). The nucleic acid sequence of interest is then stably integrated into the plant genome by infection with the transformed *Agrobacterium* strain. For example, heterologous

nucleic acid sequences have been introduced into plant tissues using the natural DNA transfer system of *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* bacteria (for review, see Klee *et al.*, Ann. Rev. Plant Phys. 38:467-486 [1987]).

There are three common methods to transform plant cells with *Agrobacterium*. The first method is co-cultivation of *Agrobacterium* with cultured isolated protoplasts. This method requires an established culture system that allows culturing protoplasts and plant regeneration from cultured protoplasts. The second method is transformation of cells or tissues with *Agrobacterium*. This method requires (a) that the plant cells or tissues can be transformed by *Agrobacterium* and (b) that the transformed cells or tissues can be induced to regenerate into whole plants. The third method is transformation of seeds, apices or meristems with *Agrobacterium*. This method requires micropropagation.

One of skill in the art knows that the efficiency of transformation by *Agrobacterium* may be enhanced by using a number of methods known in the art. For example, the inclusion of a natural wound response molecule such as acetosyringone (AS) to the *Agrobacterium* culture has been shown to enhance transformation efficiency with *Agrobacterium tumefaciens* [Shahla *et al.*, Plant Molec. Biol. 8:291-298 [1987]]. Alternatively, transformation efficiency may be enhanced by wounding the target tissue to be transformed. Wounding of plant tissue may be achieved, for example, by punching, maceration, bombardment with microprojectiles, etc. [See *e.g.*, Bidney *et al.*, Plant Molec. Biol. 18:301-313 [1992]].

In still further embodiments, the plant cells are transfected with vectors via particle bombardment (*i.e.*, with a gene gun). Particle mediated gene transfer methods are known in the art, are commercially available, and include, but are not limited to, the gas driven gene delivery instrument described in McCabe, U.S. Pat. No. 5,584,807, the entire contents of which are herein incorporated by reference. This method involves coating the nucleic acid sequence of interest onto heavy metal particles, and accelerating the coated particles under the pressure of compressed gas for delivery to the target tissue.

Other particle bombardment methods are also available for the introduction of heterologous nucleic acid sequences into plant cells. Generally, these methods involve depositing the nucleic acid sequence of interest upon the surface of small, dense particles of a material such as gold, platinum, or tungsten. The coated particles are themselves then coated onto either a rigid surface, such as a metal plate, or onto a carrier sheet made of a fragile material such as mylar. The coated sheet is then accelerated toward the target biological tissue. The use of the flat sheet generates a uniform spread of accelerated particles which maximizes the number of cells receiving particles under uniform conditions, resulting in the introduction of the nucleic acid sample into the target tissue.

Plants, plant cells and tissues transformed with a heterologous nucleic acid sequence of interest are readily detected using methods known in the art including, but not limited to, restriction mapping of the genomic DNA, PCR-analysis, DNA-DNA hybridization, DNA-RNA hybridization, DNA sequence analysis and the like.

Additionally, selection of transformed plant cells may be accomplished using a selection marker gene. It is preferred, though not necessary, that a selection marker gene be used to select transformed plant cells. A selection marker gene may confer positive or negative selection.

A positive selection marker gene may be used in constructs for random integration and site-directed integration. Positive selection marker genes include antibiotic resistance genes, and herbicide resistance genes and the like. In one embodiment, the positive selection marker gene is the NPTII gene which confers resistance to geneticin (G418) or kanamycin. In another embodiment the positive selection marker gene is the HPT gene which confers resistance to hygromycin. The choice of the positive selection marker gene is not critical to the invention as long as it encodes a functional polypeptide product. Positive selection genes known in the art include, but are not limited to, the ALS gene (chlorsulphuron resistance), and the DHFR-gene (methothrexate resistance).

A negative selection marker gene may also be included in the constructs. The use of one or more negative selection marker genes in combination with a positive

selection marker gene is preferred in constructs used for homologous recombination. Negative selection marker genes are generally placed outside the regions involved in the homologous recombination event. The negative selection marker gene serves to provide a disadvantage (preferably lethality) to cells that have integrated these genes into their genome in an expressible manner. Cells in which the targeting vectors for homologous recombination are randomly integrated in the genome will be harmed or killed due to the presence of the negative selection marker gene. Where a positive selection marker gene is included in the construct, only those cells having the positive selection marker gene integrated in their genome will survive.

The choice of the negative selection marker gene is not critical to the invention as long as it encodes a functional polypeptide in the transformed plant cell. The negative selection gene may for instance be chosen from the aux-2 gene from the Ti-plasmid of *Agrobacterium*, the *tk*-gene from SV40, cytochrome P450 from *Streptomyces griseolus*, the *Adh*-gene from Maize or *Arabidopsis*, etc. Any gene encoding an enzyme capable of converting a substance which is otherwise harmless to plant cells into a substance which is harmful to plant cells may be used.

It is contemplated that the Nod2 polynucleotides of the present invention may be utilized to either increase or decrease the level of Nod2 mRNA and/or protein in transfected cells as compared to the levels in wild-type cells. Accordingly, in some embodiments, expression in plants by the methods described above leads to the overexpression of Nod2 in transgenic plants, plant tissues, or plant cells.

In other embodiments of the present invention, the Nod2 polynucleotides are utilized to decrease the level of Nod2 protein or mRNA in transgenic plants, plant tissues, or plant cells as compared to wild-type plants, plant tissues, or plant cells.

One method of reducing Nod2 expression utilizes expression of antisense transcripts. Antisense RNA has been used to inhibit plant target genes in a tissue-specific manner (e.g., van der Krol *et al.*, Biotechniques 6:958-976 [1988]). Antisense inhibition has been shown using the entire cDNA sequence as well as a partial cDNA sequence (e.g., Sheehy *et al.*, Proc. Natl. Acad. Sci. USA 85:8805-8809 [1988]; Cannon *et al.*, Plant

Mol. Biol. 15:39-47 [1990]). There is also evidence that 3' non-coding sequence fragment and 5' coding sequence fragments, containing as few as 41 base-pairs of a 1.87 kb cDNA, can play important roles in antisense inhibition (Ch'ng *et al.*, Proc. Natl. Acad. Sci. USA 86:10006-10010 [1989]).

Accordingly, in some embodiments, the Nod2 nucleic acids of the present invention (*e.g.*, SEQ ID NOs: 1 and 33, and fragments and variants thereof) are oriented in a vector and expressed so as to produce antisense transcripts. To accomplish this, a nucleic acid segment from the desired gene is cloned and operably linked to a promoter such that the antisense strand of RNA will be transcribed. The expression cassette is then transformed into plants and the antisense strand of RNA is produced. The nucleic acid segment to be introduced generally will be substantially identical to at least a portion of the endogenous gene or genes to be repressed. The sequence, however, need not be perfectly identical to inhibit expression. The vectors of the present invention can be designed such that the inhibitory effect applies to other proteins within a family of genes exhibiting homology or substantial homology to the target gene.

Furthermore, for antisense suppression, the introduced sequence also need not be full length relative to either the primary transcription product or fully processed mRNA. Generally, higher homology can be used to compensate for the use of a shorter sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and homology of non-coding segments may be equally effective. Normally, a sequence of between about 30 or 40 nucleotides and about full length nucleotides should be used, though a sequence of at least about 100 nucleotides is preferred, a sequence of at least about 200 nucleotides is more preferred, and a sequence of at least about 500 nucleotides is especially preferred.

Catalytic RNA molecules or ribozymes can also be used to inhibit expression of the target gene or genes. It is possible to design ribozymes that specifically pair with virtually any target RNA and cleave the phosphodiester backbone at a specific location, thereby functionally inactivating the target RNA. In carrying out this cleavage, the ribozyme is not itself altered, and is thus capable of recycling and

cleaving other molecules, making it a true enzyme. The inclusion of ribozyme sequences within antisense RNAs confers RNA-cleaving activity upon them, thereby increasing the activity of the constructs.

A number of classes of ribozymes have been identified. One class of ribozymes is derived from a number of small circular RNAs which are capable of self-cleavage and replication in plants. The RNAs replicate either alone (viroid RNAs) or with a helper virus (satellite RNAs). Examples include RNAs from avocado sunblotch viroid and the satellite RNAs from tobacco ringspot virus, lucerne transient streak virus, velvet tobacco mottle virus, *Solanum nodiflorum* mottle virus and subterranean clover mottle virus. The design and use of target RNA-specific ribozymes is described in Haseloff, *et al.*, *Nature* 334:585-591 (1988).

Another method of reducing Nod2 expression utilizes the phenomenon of cosuppression or gene silencing (*See e.g.*, U.S. Pat. No. 6,063,947, incorporated herein by reference). The phenomenon of cosuppression has also been used to inhibit plant target genes in a tissue-specific manner. Cosuppression of an endogenous gene using a full-length cDNA sequence as well as a partial cDNA sequence (730 bp of a 1770 bp cDNA) are known (*e.g.*, Napoli *et al.*, *Plant Cell* 2:279-289 [1990]; van der Krol *et al.*, *Plant Cell* 2:291-299 [1990]; Smith *et al.*, *Mol. Gen. Genetics* 224:477-481 [1990]). Accordingly, in some embodiments the Nod2 nucleic acids (*e.g.*, SEQ ID NOs:1 and 33), and fragments and variants thereof are expressed in another species of plant to effect cosuppression of a homologous gene. -

Generally, where inhibition of expression is desired, some transcription of the introduced sequence occurs. The effect may occur where the introduced sequence contains no coding sequence per se, but only intron or untranslated sequences homologous to sequences present in the primary transcript of the endogenous sequence. The introduced sequence generally will be substantially identical to the endogenous sequence intended to be repressed. This minimal identity will typically be greater than about 65%, but a higher identity might exert a more effective repression of expression of the endogenous sequences. Substantially greater identity of more than about 80% is

preferred, though about 95% to absolute identity would be most preferred. As with antisense regulation, the effect should apply to any other proteins within a similar family of genes exhibiting homology or substantial homology.

For cosuppression, the introduced sequence in the expression cassette, needing less than absolute identity, also need not be full length, relative to either the primary transcription product or fully processed mRNA. This may be preferred to avoid concurrent production of some plants which are overexpressers. A higher identity in a shorter than full length sequence compensates for a longer, less identical sequence. Furthermore, the introduced sequence need not have the same intron or exon pattern, and identity of non-coding segments will be equally effective. Normally, a sequence of the size ranges noted above for antisense regulation is used.

VIII. Drug Screening Using Nod2

The present invention provides methods and compositions for using Nod2 as a target for screening drugs that can alter, for example, RICK signalling, and thus the physiological effects of NF- κ B (*e.g.*, inflammatory response). For example, drugs that induce or inhibit NF- κ B mediated inflammatory responses can be identified by screening for compounds that target Nod2 or regulate Nod2 gene expression.

The present invention is not limited to a particular mechanism of action. Indeed, an understanding of the mechanism of action is not necessary to practice the present invention. Nevertheless, it is contemplated that Nod2 binds to RICK, and this binding results in the activation on NF- κ B. Accordingly, it is contemplated that binding assays are useful for screening for compounds that block Nod2 binding to RICK. In particular, it is contemplated that such screens are capable of identifying compounds that are useful for inhibiting NF- κ B activity and thus for treating Crohn's disease. The binding need not employ full-length RICK and Nod2. Indeed, portions of RICK and Nod2 may be utilized in the binding assays. For example, in some embodiments, a fragment of Nod2 containing the two CARD domains is utilized in the binding assay.

In one screening method, the two-hybrid system is used to screen for compounds (*e.g.*, drug) capable of altering (*e.g.*, inhibiting) Nod2 function(s) (*e.g.*, NF- κ B-mediated signal transduction) *in vitro* or *in vivo*. In one embodiment, a GAL4 binding site, linked to a reporter gene such as *lacZ*, is contacted in the presence and absence of a candidate compound with a GAL4 binding domain linked to a Nod2 fragment and a GAL4 transactivation domain II linked to a NF- κ B fragment. Expression of the reporter gene is monitored and a decrease in the expression is an indication that the candidate compound inhibits the interaction of Nod2 with NF- κ B. Alternately, the effect of candidate compounds on the interaction of Nod2 with other proteins (*e.g.*, proteins known to interact directly or indirectly with NF- κ B) can be tested in a similar manner.

In another screening method, candidate compounds are evaluated for their ability to alter Nod2 signalling by contacting Nod2, NF- κ B, NF- κ B-associated proteins, or fragments thereof, with the candidate compound and determining binding of the candidate compound to the peptide. The protein or protein fragments is/are immobilized using methods known in the art such as binding a GST-Nod2 fusion protein to a polymeric bead containing glutathione. A chimeric gene encoding a GST fusion protein is constructed by fusing DNA encoding the polypeptide or polypeptide fragment of interest to the DNA encoding the carboxyl terminus of GST (*See e.g.*, Smith *et al.*, Gene 67:31 [1988]). The fusion construct is then transformed into a suitable expression system (*e.g.*, *E. coli* XA90) in which the expression of the GST fusion protein can be induced with isopropyl- β -D-thiogalactopyranoside (IPTG). Induction with IPTG should yield the fusion protein as a major constituent of soluble, cellular proteins. The fusion proteins can be purified by methods known to those skilled in the art, including purification by glutathione affinity chromatography. Binding of the candidate compound to the proteins or protein fragments is correlated with the ability of the compound to disrupt the signal transduction pathway and thus regulate Nod2 physiological effects (*e.g.*, apoptosis).

In another screening method, one of the components of the Nod2/NF- κ B signalling system, such as Nod2 or a fragment of Nod2, is immobilized. Polypeptides can be immobilized using methods known in the art, such as adsorption onto a plastic microtiter plate or specific binding of a GST-fusion protein to a polymeric bead containing glutathione. For example, GST-Nod2 is bound to glutathione-Sepharose beads. The immobilized peptide is then contacted with another peptide with which it is capable of binding in the presence and absence of a candidate compound. Unbound peptide is then removed and the complex solubilized and analyzed to determine the amount of bound labeled peptide. A decrease in binding is an indication that the candidate compound inhibits the interaction of Nod2 with the other peptide. A variation of this method allows for the screening of compounds that are capable of disrupting a previously-formed protein/protein complex. For example, in some embodiments a complex comprising Nod2 or a Nod2 fragment bound to another peptide is immobilized as described above and contacted with a candidate compound. The dissolution of the complex by the candidate compound correlates with the ability of the compound to disrupt or inhibit the interaction between Nod2 and the other peptide.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to Nod2 peptides and is described in detail in WO 84/03564, incorporated herein by reference. Briefly, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are then reacted with Nod2 peptides and washed. Bound Nod2 peptides are then detected by methods well known in the art.

Another technique uses Nod2 antibodies, generated as discussed above. Such antibodies capable of specifically binding to Nod2 peptides compete with a test compound for binding to Nod2. In this manner, the antibodies can be used to detect the presence of any peptide that shares one or more antigenic determinants of the Nod2 peptide.

In some embodiments of the present invention, compounds are screened for their ability to inhibit the binding of pathogen components (e.g., including, but not limited to, bacterial cell surface proteins; fungi proteins, parasite proteins, and virus proteins) to Nod2. Any suitable screening assay may be utilized, including, but not limited to, those described herein.

The present invention contemplates many other means of screening compounds. The examples provided above are presented merely to illustrate a range of techniques available. One of ordinary skill in the art will appreciate that many other screening methods can be used.

In particular, the present invention contemplates the use of cell lines transfected with Nod2 and variants or mutants thereof for screening compounds for activity, and in particular to high throughput screening of compounds from combinatorial libraries (e.g., libraries containing greater than 10^4 compounds). The cell lines of the present invention can be used in a variety of screening methods. In some embodiments, the cells can be used in second messenger assays that monitor signal transduction following activation of cell-surface receptors. In other embodiments, the cells can be used in reporter gene assays that monitor cellular responses at the transcription/translation level. In still further embodiments, the cells can be used in cell proliferation assays to monitor the overall growth/no growth response of cells to external stimuli.

In second messenger assays, the host cells are preferably transfected as described above with vectors encoding Nod2 or variants or mutants thereof. The host cells are then treated with a compound or plurality of compounds (e.g., from a combinatorial library) and assayed for the presence or absence of a response. It is contemplated that at least some of the compounds in the combinatorial library can serve as agonists, antagonists, activators, or inhibitors of the protein or proteins encoded by the vectors. It is also contemplated that at least some of the compounds in the combinatorial library can serve as agonists, antagonists, activators, or inhibitors of protein acting upstream or downstream of the protein encoded by the vector in a signal transduction pathway.

In some embodiments, the second messenger assays measure fluorescent signals from reporter molecules that respond to intracellular changes (*e.g.*, Ca^{2+} concentration, membrane potential, pH, IP_3 , cAMP, arachidonic acid release) due to stimulation of membrane receptors and ion channels (*e.g.*, ligand gated ion channels; *see* Denyer *et al.*, Drug Discov. Today 3:323-32 [1998]; and Gonzales *et al.*, Drug. Discov. Today 4:431-39 [1999]). Examples of reporter molecules include, but are not limited to, FRET (fluorescence resonance energy transfer) systems (*e.g.*, Cuo-lipids and oxonols, EDAN/DABCYL), calcium sensitive indicators (*e.g.*, Fluo-3, FURA 2, INDO 1, and FLUO3/AM, BAPTA AM), chloride-sensitive indicators (*e.g.*, SPQ, SPA), potassium-sensitive indicators (*e.g.*, PBFI), sodium-sensitive indicators (*e.g.*, SBFI), and pH sensitive indicators (*e.g.*, BCECF).

In general, the host cells are loaded with the indicator prior to exposure to the compound. Responses of the host cells to treatment with the compounds can be detected by methods known in the art, including, but not limited to, fluorescence microscopy, confocal microscopy (*e.g.*, FCS systems), flow cytometry, microfluidic devices, FLIPR systems (*See, e.g.*, Schroeder and Neagle, J. Biomol. Screening 1:75-80 [1996]), and plate-reading systems. In some preferred embodiments, the response (*e.g.*, increase in fluorescent intensity) caused by compound of unknown activity is compared to the response generated by a known agonist and expressed as a percentage of the maximal response of the known agonist. The maximum response caused by a known agonist is defined as a 100% response. Likewise, the maximal response recorded after addition of an agonist to a sample containing a known or test antagonist is detectably lower than the 100% response.

The cells are also useful in reporter gene assays. Reporter gene assays involve the use of host cells transfected with vectors encoding a nucleic acid comprising transcriptional control elements of a target gene (*i.e.*, a gene that controls the biological expression and function of a disease target) spliced to a coding sequence for a reporter gene. Therefore, activation of the target gene results in activation of the reporter gene product. As described above, it is contemplated that Nod2 binds to

10014269-102601
5 RICK, and this binding results in the activation on NF- κ B. Therefore, in some
embodiments, the reporter gene construct comprises the 5' regulatory region (*e.g.*,
promoters and/or enhancers) of a protein whose expression is controlled by NF- κ B in
operable association with a reporter gene (See Example 4 and Inohara et al., J. Biol.
Chem. 275:27823-31 [2000] for a description of the luciferase reporter construct
pBVix-Luc). Examples of reporter genes finding use in the present invention include,
but are not limited to, chloramphenicol transferase, alkaline phosphatase, firefly and
bacterial luciferases, β -galactosidase, β -lactamase, and green fluorescent protein. The
production of these proteins, with the exception of green fluorescent protein, is
10 detected through the use of chemiluminescent, colorimetric, or bioluminescent products
of specific substrates (*e.g.*, X-gal and luciferin). Comparisons between compounds of
known and unknown activities may be conducted as described above.

IX. Pharmaceutical Compositions Containing Nod2 Nucleic Acid, Peptides, and Analog

15 The present invention further provides pharmaceutical compositions which may
comprise all or portions of Nod2 polynucleotide sequences, Nod2 polypeptides,
inhibitors or antagonists of Nod2 bioactivity, including antibodies, alone or in
combination with at least one other agent, such as a stabilizing compound, and may be
administered in any sterile, biocompatible pharmaceutical carrier, including, but not
20 limited to, saline, buffered saline, dextrose, and water.

The methods of the present invention find use in treating diseases or altering
physiological states characterized by apoptosis of cells or other NF- κ B mediated
effects. The invention provides methods for inhibiting Nod2 interaction with NF- κ B
and NF- κ B-associated proteins by administering peptides or peptide fragments of
25 Nod2. Peptides can be administered to the patient intravenously in a pharmaceutically
acceptable carrier such as physiological saline. Standard methods for intracellular
delivery of peptides can be used (*e.g.*, delivery via liposome). Such methods are well
known to those of ordinary skill in the art. The formulations of this invention are
useful for parenteral administration, such as intravenous, subcutaneous, intramuscular,

and intraperitoneal. Therapeutic administration of a polypeptide intracellularly can also be accomplished using gene therapy as described above.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and interaction with other drugs being concurrently administered.

Accordingly, in some embodiments of the present invention, Nod2 nucleotide and Nod2 amino acid sequences can be administered to a patient alone, or in combination with other nucleotide sequences, drugs or hormones or in pharmaceutical compositions where it is mixed with excipient(s) or other pharmaceutically acceptable carriers. In one embodiment of the present invention, the pharmaceutically acceptable carrier is pharmaceutically inert. In another embodiment of the present invention, Nod2 polynucleotide sequences or Nod2 amino acid sequences may be administered alone to individuals subject to or suffering from a disease.

Depending on the condition being treated, these pharmaceutical compositions may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in the latest edition of "Remington's Pharmaceutical Sciences" (Mack Publishing Co, Easton Pa.). Suitable routes may, for example, include oral or transmucosal administration; as well as parenteral delivery, including intramuscular, subcutaneous, intramedullary, intrathecal, intraventricular, intravenous, intraperitoneal, or intranasal administration.

For injection, the pharmaceutical compositions of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. For tissue or cellular administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

In other embodiments, the pharmaceutical compositions of the present invention can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical

compositions to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral or nasal ingestion by a patient to be treated.

Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. For example, an effective amount of Nod2 may be that amount that suppresses apoptosis. Determination of effective amounts is well within the capability of those skilled in the art, especially in light of the disclosure provided herein.

In addition to the active ingredients these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions.

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known (*e.g.*, by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes).

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to

obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, etc; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxymethylcellulose; and gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, (*i.e.*, dosage).

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Compositions comprising a compound of the invention formulated in a pharmaceutical acceptable carrier may be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. For polynucleotide or amino acid sequences of Nod2, conditions indicated on the label may include treatment of condition related to apoptosis.

The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, *etc.* Salts tend to be more soluble in aqueous or other protonic solvents that are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder in 1 mM-50 mM histidine,

0.1%-2% sucrose, 2%-7% mannitol at a pH range of 4.5 to 5.5 that is combined with buffer prior to use.

For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. Then, preferably, dosage can be formulated in animal models (particularly murine models) to achieve a desirable circulating concentration range that adjusts Nod2 levels.

A therapeutically effective dose refers to that amount of Nod2 which ameliorates symptoms of the disease state. Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD₅₀ (the dose lethal to 50% of the population) and the ED₅₀ (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio LD₅₀/ED₅₀. Compounds which exhibit large therapeutic indices are preferred. The data obtained from these cell culture assays and additional animal studies can be used in formulating a range of dosage for human use. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED₅₀ with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

The exact dosage is chosen by the individual physician in view of the patient to be treated. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Additional factors which may be taken into account include the severity of the disease state; age, weight, and gender of the patient; diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long acting pharmaceutical compositions might be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature (*See*, U.S. Pat.

Nos. 4,657,760; 5,206,344; or 5,225,212, all of which are herein incorporated by reference). Those skilled in the art will employ different formulations for Nod2 than for the inhibitors of Nod2. Administration to the bone marrow may necessitate delivery in a manner different from intravenous injections.

5 **EXPERIMENTAL**

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

10 In the experimental disclosure which follows, the following abbreviations apply: eq (equivalents); M (Molar); μ M (micromolar); N (Normal); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); g (grams); mg (milligrams); μ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); $^{\circ}$ C (degrees Centigrade); U (units), mU (milliunits); min. (minutes); sec. (seconds); % (percent); kb (kilobase); bp (base pair); PCR (polymerase chain reaction); 15 BSA (bovine serum albumin); Fisher (Fisher Scientific, Pittsburgh, PA); Sigma (Sigma Chemical Co., St. Louis, MO.); Promega (Promega Corp., Madison, WI); Perkin-Elmer (Perkin-Elmer/Applied Biosystems, Foster City, CA); Boehringer Mannheim (Boehringer Mannheim, Corp., Indianapolis, IN); Clonetech (Clonetech, Palo Alto, CA); 20 Qiagen (Qiagen, Santa Clarita, CA); Stratagene (Stratagene Inc., La Jolla, CA); National Biosciences (National Biosciences Inc, Plymouth Minn.) and NEB (New England Biolabs, Beverly, MA), CARD (caspase-recruitment domain); EST (expressed sequence tag); HA (hemagglutinin); I κ B (inhibitor of NF- κ B); IKK (I κ B kinase); LRRs (leucine-rich repeats); NBD (nucleotide-binding domain); NF- κ B (nuclear factor κ B); 25 TNF α (tumor necrosis factor α); wt (wild-type); Ab (antibody); IL-1 (interleukin 1); IL-1R (IL-1 receptor); LPS (lipopolysaccharide); LTA (lipoteichoic acid); PGN (peptidoglycan); SBLP (synthetic bacterial lipoprotein); and TLR (Toll-like receptor).

METHODOLOGY

Reagents. LPS from various sources in this study were obtained from Sigma (St. Louis, MO). PGN from *Staphylococcus aureus* was obtained from Fluka-Chemie (Buchs, Germany). Mannan from *Candida albicans* 20A was a gift of P. Lehmann (Medical College of Ohio). PaM3CysSerLyS4, a synthetic bacterial lipoprotein analogue (SBLP) was a gift of A. Zychlinsky (New York University School of Medicine).

Isolation of the Nod2 cDNA. Nucleotide sequences encoding peptides with homology to Nod1 (GeneBank accession numbers AC007728 and AQ534686) were found in the public genomic database using the TBLASTN program. The coding region of human nod2 was obtained by reverse transcriptase (RT)-PCR amplification and 5' RACE using Nod2-specific oligonucleotide primers cDNA fragments and MRNA from primary mammary tissue as a template. 5' RACE was performed using a commercial kit (Roche Molecular Biochemicals, Indianapolis, IN). For PCR, three sets of primers were used: 5'-ATGTGCTCGCAGGAGGCTTTTCAGGCA-3' (SEQ ID NO:37) and 5'-CGCCTCACCCACCACCAGCACAGTGT-3' (SEQ ID NO:38); 5'-CATGGCTGGACCCCCGCAGAAGAGCCCA-3' (SEQ ID NO:39) and 5'-CATGCCCGGGTTCATCTGGCTCATCCGG-3' (SEQ ID NO:40); 5'-GCCATGCCCGGGTTCATCTGGCTCATC-3' (SEQ ID NO:41) and 5'-TGAGTCGAGACATGGGGAAAGCTGCTTC-3' (SEQ ID NO:42). For 5' RACE, the initial primer 5'-AGCAGCTCGACCAGCTGGCTCCTCTGT-3' (SEQ ID NO:43) was used and the product was PCR amplified with the anchored primer and second Nod2-specific primer: 5'-GACAGGCCCAAGTACCCTTATTCCAGA-3' (SEQ ID NO:44). The resulting cDNA fragments were digested with restriction enzymes and ligated to generate an unique cDNA containing the entire open reading frame of Nod2. The cDNA sequence was verified by nucleotide sequencing.

Northern Blot and RT-PCR Analysis of Nod2 Expression. A 3.7 kb fragment containing the entire Nod2 coding region was radiolabeled by random priming using a commercial kit (Roche Molecular Biochemicals) and applied for analysis of human poly(A)' RNA blots from various tissues (Clontech Laboratories, Palo Alto, CA)

according to the manufacturer's instructions. Peripheral blood leukocytes were obtained from heparinized venous blood from healthy volunteers by Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. Granulocytes were separated from red blood cells by brief incubation with hypotonic lysis buffer.

5 The mononuclear cell population was fractionated into lymphocytes and monocytes by adherence to plastic dishes. For RT-PCR analysis, 2 µg of total RNA from each cell preparations were used to generate first strand cDNA using a commercially available kit (Gibco BRL; Gaithersburg, MD). Nod2 cDNA fragments corresponding to the Nod2 coding region were amplified by PCR using two sets of specific primers; P1: 10 5'-ATGTGCTCGCAGGAGGCTTTTCAGGCA-3' (SEQ ID NO:45); P2: 5'-CGCCTCACCCACCACCAGCACAGTGT-3' (SEQ ID NO:46); P3: 5'-ATGTGCTCGCAGGAGGCTTTTCAGGCA-3' (SEQ ID NO:47) and P4: 5'-CG-CCTCACCCACCACCAGCACAGTGT-3' (SEQ ID NO:48). As a control, a CDNA fragment of the human glyceraldehyde-3 -phosphate dehydrogenase was amplified 15 using the primers 5'-GAGTCAACGGATTTGGTCGTAT-3' (SEQ ID NO:49) and 5'-AGTCTTCTGGGTGGCAGTGAT-3' (SEQ ID NO:50).

Construction of Expression Plasmids. The Nod2 cDNA was cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara *et al.*, [2000], *supra*). Deletion and sitedirected mutants of Nod2 (129-1040, A125-214, 1-125, 1-301, 1-744, 265-1040, 20 126-301, 265744, 744-1040, K305R, 1-744K305R) were constructed by a PCR method and cloned into pcDNA3-HA and pcDNA3-Fpk3-Myc (Inohara *et al.*, [2000], *supra*). The authenticity of all constructs was confirmed by sequencing. pcDNA3-Flag-RICK, pcDNA3-Flag-RICK(1-374), pcDNA3-Flag-RICK(374-540), pcDNA3-Myc-RICK(406-540), pcDNA3-Myc-RIP(558-671), pRK7-Flag-IKKα, 25 pRK7-FlagIKKα-K44A, RSVMad-3MSS(Iκ-Bα-S32A/S36A), pRK7-Flag-IKKβ, pRK7-Flag-IKKβ-K44A, and pcDNA3-Flag-IKKγ(134-419) have been described previously (Inohara *et al.*, *supra*, 10). The expression plasmids pcDNA3-Nod1-Flag, pcDNA3-Nod1 (I -648)-Flag, pcDNA3-Flag-IKKi, pcDNA3CIPER-Flag, pCMV-ILIR, pCMV-TLR4-Flag, pcDNA3-Flag-RIP, pcDNA3-MyD88 DN(amino acids 1-109), 30 pcDNA3-CD14, pCMV-MD2-FLAG and pcDNA3-β-gal have also been described

previously (Inohara *et al.*, [1999], *Supra*; Inohara *et al.*, [1999], *Supra*; Inohara *et al.*, [2000], *supra*; Shimada *et al.*, *Int. Immunol.*, 11:1357-1362 [1999]; Huang *et al.*, *PNAS*, 94:12829-12832 [1997]; Medzhitov *et al.*, *Mol. Cell*, 2:253-258 [1998]; Hsu *et al.*, *Immunity*, 4:387-396 [1996]). To construct the expression plasmid producing C-terminally HA-tagged mature interleukin-1 P (IL1 β), pcDNA3-mIL1 β -HA, the mature region of mouse IL1 β was amplified by PCR and inserted into pcDNA3-HA-pro which contains the signal sequence of protrypsin and the HA tag.

Transfection, Expression, Immunoprecipitation and Immunodetection of Tagged Proteins. HEK293T cells were co-transfected with pcDNA3-Nod2-HA and various expression plasmids as described (Inohara *et al.*, [1999] *supra*). To test the interaction between wt RICK and Nod2 mutant proteins, HEK293T cells were co-transfected with pcDNA3-Flag-RICK and wt or mutant Nod2 expression plasmids. Proteins co-immunoprecipitated with anti-HA antibody were detected with anti-Flag antibody. To test the interaction between wt Nod2 and RICK mutants, HEK293T cells were cotransfected with pcDNA3-HA-Nod2 and pcDNA3-Flag-RICK, pcDNA3-Flag-RICK(1-374) or pcDNA3-Flag-RICK(374-540) (Inohara *et al.*, [1999] *supra*). Proteins co-immunoprecipitated with anti-HA antibody were detected with anti-Flag antibody. Proteins in total lysate were detected by anti-Flag and anti-HA monoclonal antibody, respectively.

NF- κ B activation assays. NF- κ B activation assays were performed as described (Inohara *et al.*, [1999] *supra*, Inohara *et al.*, [2000], *supra*). Briefly, Rat1 fibroblasts and its derivative 5R cell line (Yamaoka *et al.*, *Cell* 93: 1231-1240 [1998]) as well as HEK293T cells were co-transfected with 12 ng of the reporter construct pBVIx-Luc, plus indicated amounts of each expression plasmid and 120 ng of pEF-BOS- β -gal in triplicate as described. 24 hr post-transfection, cell extracts were prepared and its relative luciferase activity was measured as described (Inohara *et al.*, [1999] *supra*, Inohara *et al.*, [2000], *supra*). Results were normalized for transfection efficiency with values obtained with pEF-BOS- β -gal.

In vitro LPS binding assay. 1×10^8 HEK293T cells were transfected with expression plasmids indicated in figure legends as described (Inohara *et al.*, [2000], *Supra*). Twenty-four hr post-transfection, S100 fractions were prepared from transfected cells as described using Buffer A (Poltorak *et al.*, [1998], *Supra*). For Fig. 5 10A, S100 lysate containing 5 mg of protein was incubated with 300 ng [3 H] LPS (1×10^5 Bq, 347 Bq/ng, List Biological Laboratories, Campbell, CA) from *Escherichia coli* K12 KCD25, 6 μ g anti-FLAG M2 antibody (Sigma Chemical), 10 μ l Protein A-Sepharose and 10 μ l Protein G-Sepharose at 4°C for 2 hr. Proteins bound to the matrix were washed 5 times with 1 ml of Buffer A. The bound radioactivity was 10 measured using a Liquid Scintillation Counter Beckman LS5000LD. For Fig. 3B, proteins were immunopurified first from 20 mg of S100 lysate as described above and incubated with 300 ng [3 H] LPS in the presence of 10 mg bovine serum albumin Fraction V (Sigma Chemical) at 4°C for 2 hr. After 5 washes with 1 ml of Buffer A, the bound radioactivity was measured. To monitor protein expression, proteins in 50 15 μ g of S100 lysate were detected by immunoblotting with anti-FLAG Ab.

Example 1

This Example describes the identification of Nod2. To identify novel Nod1/Apaf- I -like molecules, public genomic data bases were searched for genes encoding proteins with homology to Nod1 (Inohara *et al.*, *supra*). A genomic sequence 20 was identified in human chromosome 16 (GeneBank accession number AC007728) that encodes a peptide with significant homology to the NBD of Nod1. Analysis with GeneFinder of the genomic region predicted a gene encoding a novel protein with significant homology to Nod1. To determine the ends of the coding region, 5' RACE was performed using an oligonucleotide complementary to sequences encoding the 25 N-terminus of the predicted protein and sequenced several EST cDNAs which contain partial sequences of the gene (GeneBank accession numbers AA775466, AA910520, A1090427). To amplify the cDNA containing the entire open reading frame, we RT-PCR was performed with three sets of primers corresponding to overlapping sequences

of the coding region of the gene. The predicted open reading frame encodes a protein of 1040 amino acids. A BLAST search of protein data bases indicated that the protein encoded by the new open reading frame was most homologous to Nod1 (34% amino acid identity). This protein was designated Nod2 given its high level of homology with Nod1 and thus represents a novel member of the Apaf-1/Nod1 superfamily (Fig. 1). Analysis of the nucleotide sequence revealed two potential in-frame translation initiation sites separated by 81 nucleotides. Further analysis revealed that both translation initiation sites can be utilized in cells, although the longer open reading frame is preferentially used (see below). For simplicity, the longer open reading frame is designated Nod2 and the product encoded by the shorter open reading frame is designated as Nod2b. A BLAST search and domain analyses revealed that Nod2 is composed of two NH₂-terminal CARDs (residues 28-220) fused to a centrally located NBD domain (residues 273-577) containing consensus nucleotide-binding motifs followed by ten tandem LRRs (residues 744-1020) (Figs. 1 and 2). Each of the 10 LRRs of Nod2 contained predicted α helix and β sheet sequences that is consistent with the prototypical horseshoe-shaped structure of LRRs (Kobe and Deisenhofer, Curr. Opin. Struct. Biol. 5: 409-416 [1995]) (Fig. 2C). Nod2 is the first protein known to encode two CARDs.

Example 2

This Example describes the chromosomal localization and genomic organization of the human Nod2 gene. Two human BAC clones, RPII-327F22 and RPII-40IP9, containing the genomic sequence of human Nod2 (GenBank accession numbers AC007728 and AC007608, respectively) were identified. These BAC clones mapped to chromosome 16 at q12. Comparison of Nod2 cDNA and genomic sequences revealed that the Nod2 gene contains twelve coding exons.

Example 3

This Example demonstrates that the expression of Nod2 is most abundant in monocytes. Northern blot analysis showed Nod2 to be expressed as two 7.0 and 5.5

kb transcripts in peripheral blood leukocytes with little or no detectable expression in various human tissues (Fig. 3A). This highly restricted pattern of expression is in contrast to that of Nod1 and Apaf-1 which are expressed in virtually all adult tissues although at different levels (Inohara *et al.*, *supra*). To determine the cells that express Nod2, peripheral blood leukocytes were fractionated into granulocyte, lymphocyte and monocyte populations and analyzed by RT-PCR analysis with two different sets of oligonucleotide primers complementary to Nod2 coding sequences. The analysis showed that Nod2 was expressed primarily in monocytes (Fig. 3B). Because the Nod2 sequence contained two potential in-frame translation initiation sites separated by 81 nucleotides (Fig. 3C), their usage was determined by transfection of a Nod2 construct containing both translation initiation sites into HEK293T cells. Because the difference in size between both predicted Nod2 products is only 27 amino acids, we expressed a COOH-terminally truncated Nod2 lacking residues 302-1040 to facilitate the identification of the translation initiation sites. As a control, Nod2 plasmids were engineered that express each translation initiation site separately within a canonical Kozak's translation initiation motif. The analysis revealed that both translation initiation sites in the Nod2 open reading frame were used, although the most NH2-terminal translation initiation codon was more efficient as assessed by immunoblotting of cell extracts with an antibody that recognizes a COOH-terminal HA tag (Fig. 3D).

Example 4

This example describes the activation of NF- κ B by Nod2. Because of the homology between Nod1 and Nod2, tests were conducted to determine whether expression of Nod2 activates NF- κ B by transfection of Nod2 plasmids into HEK293T cells. Transfection of the wt Nod2 cDNA induced potent activation of NF- κ B, as measured with a reporter luciferase construct (see below). In addition, we tested the Nod2b cDNA and obtained similar results to those observed with Nod2. A panel of Nod2 mutants was generated to determine the regions of Nod2 that are required for NF- κ B activation (Fig. 4A). Immunoblotting analysis revealed that these mutant

constructs were expressed when transiently transfected into HEK293T cells (Fig. 4B). Expression of as little as 3 ng of wt Nod2 induced 18-fold activation of NF- κ B (Fig. 4C). Expression of a Nod2 mutant form lacking the LRRs resulted in enhanced NF- κ B activation, while mutants expressing the LRRs or the NBD alone were inactive (Fig. 4C). The enhanced activity of the Nod2 mutant lacking the LRRs could not be explained by increased expression of the mutant (Fig. 4A). Consistent with these results, it was shown previously that deletion of the LRRs of Nod1 and WD-40 repeats of Apaf-1 results in enhanced NF- κ B activation and increased ability to activate procaspase-9, respectively (Inohara *et al.*, *supra*, Srinivasula *et al.*, *supra*, Hu *et al.*, *supra*). Deletion of the CARDs of Nod2, either singly or in combination, resulted in total loss of NF- κ B activity (Fig. 4C). However, expression of both CARDs alone, but not each CARD separately, was sufficient for NF- κ B activation (Fig. 4C). Thus, both CARDs of Nod2 are necessary and sufficient for NF- κ B activation, suggesting that the CARDs acts as an effector domain in Nod2 signaling. The conserved lysine residue in the P-loop of Nod1 and Apaf-1 is important for the activities of these proteins (Inohara *et al.*, [1999] *supra*, Inohara *et al.*, [2000], *supra*, Hu *et al.*, EMBO J. 18: 3586-3595 [1999]). Similarly, replacement of the corresponding lysine for arginine in Nod2 resulted in diminished NF- κ B activity that was rescued at least in part by deletion of the LRRs (Fig. 4C).

The ability of Nod2 to induce apoptosis was also investigated. Overexpression of Nod2 did not induce apoptosis by itself but enhanced apoptosis induced by caspase-9 expression. These results are similar to those reported for Nod1 and Apaf-1 (Bertin *et al.*, *supra*, Inohara *et al.*, [1999] *supra*).

Example 5

This example demonstrates that NF- κ B activation induced by Nod2 requires IKK γ and is inhibited by dominant negative forms of IKKs and RICK. A main pathway of NF- κ B activation is mediated by I κ B kinases (IKKS) resulting in I κ B phosphorylation and release of cytoplasmic NF- κ B (Karin, J. Biol. Chem. 274: 27339-27342 [1999]). To determine whether Nod2 activates an IKK-dependent

pathway, Nod2 was co-expressed with mutant forms of IKK α , IKK β , and I κ B that have been shown to act as dominant inhibitors of their corresponding endogenous counterparts and/or the IKK complex (Karin, *supra*). In addition, a truncated mutant of IKK γ /Nemo (residues 134-419) was used that is defective in IKK α and IKK β binding and acts as an inhibitor of NF- κ B activation induced by RIP and RICK (Inohara *et al.*, [2000], *supra*). The NF- κ B activity induced by Nod2 as well as that induced by TNF α stimulation were greatly inhibited by mutant IKK α , IKK β , IKK γ , and I κ B α (Fig. 5A). Because RICK has been shown to serve as a downstream target of Nod1 (Bertin *et al.*, *supra*, Inohara *et al.*, [1999] *supra*, Inohara *et al.*, [2000], *supra*), a truncated form of RICK containing its CARD (residues 406-540) that acts as a dominant inhibitor of Nod1 activity (Bertin *et al.*, *supra*) was used to test whether NF- κ B activation induced by Nod2 is similarly inhibited by this RICK mutant. NF- κ B activation induced by Nod2 was inhibited by mutant RICK but not by a mutant form of RIP that expresses its death effector domain (Fig. 5A). The inhibition by the CARD of RICK was specific in that it did not interfere with ability of TNF α to induce NF- κ B, an activity that was inhibited by the RIP mutant (Fig. 5A). To verify that Nod2 acts upstream of the IKK complex to activate NF- κ B, we tested the ability of Nod2 to activate NF- κ B in parental Rat1 fibroblasts and 5R cells, a Rat1 derivative cell line that is defective in IKK γ , an essential subunit of the IKKs (Yamaoka *et al.*, *supra*). Nod2, as well as Nod1 and TNF α , induced NF- κ B activity in parental Rat1 cells but not in IKK γ -deficient 5R cells (Fig. 5B). As a control, expression of IKK β , which functions downstream of IKK γ , induced NF- κ B activation in both Rat1 and 5R cell lines (Fig. 5B). These results indicate that Nod2 acts through IKK γ /IKK/IKK β to activate NF- κ B.

Example 6

This Example demonstrates that Nod2 associates with RICK via a homophilic CARD-CARD interaction. The CARD motif functions as an effector domain that mediates specific homophilic interaction with downstream CARD-containing molecules

(Hofmann *et al.*, Trends Biochem. Sci. 22: 155-156 [1997]). Because NF- κ B activation induced by Nod2 was inhibited by a RICK truncated mutant, the ability of RICK to act as a direct downstream mediator of Nod2 signaling was tested. To test a physical association between Nod2 and RICK, HEK293T cells were co-transfected with plasmids expressing HA-tagged wt or mutant forms of Nod2 and Flag-tagged RICK and cellular extracts were immunoprecipitated with anti-HA antibody. Immunoblotting with anti-Flag antibody revealed that RICK associated with Nod2 (Fig. 6A). The association was mediated by both CARDs of Nod2, as only Nod2 proteins containing both CARDs were capable of interacting with RICK (Fig. 6A, B). The association of Nod2 with RICK was specific in that Nod2 did not associate with several CARD-containing proteins including Apaf-1, caspase-1, caspase-4, c-IAP-1, c-IAP2, procaspase-9, Bcl-10, RAIDD, and Ced-4 nor with several molecules that activate NF- κ B including TRAF-1, TRAF-2, TRAF-5, TRAF-6, RIP, NIK, TRADD, IKK α , IKK β or IKK γ . To determine the region of RICK that associates with Nod2, mutant forms of RICK expressing the CARD (residues 374-540) or lacking the CARD (residues 1-374) were co-expressed with Nod2 and the cell extracts were immunoprecipitated with anti-Flag antibody. The analysis showed that only the CARD of RICK co-immunoprecipitated with Nod2 (Fig. 6C). Thus, Nod2 and RICK associate via a homophilic CARD-CARD interaction.

Example 7

This Example demonstrates that enforced oligomerization of Nod2 induces NF-KB activation. Previous studies showed that the NBD of Nod1 and Apaf-I mediates oligomerization of these molecules, an activity that is critical for NF- κ B and caspase-9 activation, respectively (Srinivasula *et al.*, *supra*, Hu *et al.*, [1998] *supra*, Inohara *et al.*, [2000], *supra*). In the case of Nod1, its oligomerization appears to promote proximity of RICK and NF- κ B activation. To test a similar role for Nod2, plasmids were constructed to express chimeric proteins in which wt or Nod2 mutants were fused to three tandem repeated dimerization domains of Fpk (Fpk3), which can be oligomerized by the cell-permeable ligand AP1510 (MacCorkle *et al.*, Proc. Nat.

Acad. Sci. U. S. A. 95: 3655-3660 [1998]). Immunoblotting analysis showed that the chimeric Fpk3-Nod2 constructs were expressed when transfected in HEK293T cells (Fig. 7A). Because wt Nod2 alone induces NF- κ B activation, we expressed suboptimal amounts of the chimeric Fpk3-Nod2 constructs into HEK293T cells. Under these experimental conditions, expression of Nod2-Fpk3 induced NF- κ B activation in a ligand-dependent manner (Fig. 7B). Consistent with the results shown in Fig. 4C, enforced oligomerization of both CARDS but not each CARD singly induced NF- κ B activation (Fig. 7B). Similarly, NF- κ B activation induced by a Nod2 P-loop mutant lacking the LRRs (K305RALRR), which have reduced ability to induce NF- κ B activation, was enhanced by enforced oligomerization (Fig. 7C). A Nod2-Fpk3 construct lacking the LRRs induced NF- κ B activation in the absence and presence of AP1510 (Fig. 7B). The latter result might be explained by our observations that Nod2 lacking the LRRs has enhanced activity to self-associate and induce NF κ B (Fig. 4C).

Example 8

This Example describes the role of Nod1 in the cellular response to microbial components. Human embryonic kidney HEK293T cells were transiently co-transfected with a Nod1 expression plasmid or control plasmid and a NF- κ B reporter construct in the presence of bacterial or fungal products. No significant induction of reporter gene activity was observed when the cells transfected with control plasmid were exposed to LPS, PGN, LTA, synthetic bacterial lipopeptide (SBLP) or mannan (Fig. 8A). These results are in agreement with previous observations in HEK293 cells (Yang *et al.*, Nature, 395:284-288 [1998]; Aliprantis *et al.*, *Supra*; Chow *et al.*, *Supra*; Schwandner *et al.*, J. Biol. chem., 274:17406-17409 [2000]). Because overexpression of Nod1 induces NF- κ B activation (Zou *et al.*, Cell, 90:405-413 [1997]; Bertin *et al.*, J. Biol. Chem., 274:12955-12858 [1999]), HEK293T cells were transfected with 0.3 ng of Nod1 and measured for NF- κ B activation after incubation with various pathogen components. LPS, but not the other microbial products tested, induced significant NF- κ B activation (about 12-fold) in cells expressing trace amounts of Nod1 (Fig. 8A). To demonstrate that NF- κ B activation by LPS is specific for cells expressing Nod1,

HEK293T cells were transfected with expression plasmids producing interleukin-1 receptor (IL1R) and its ligand interleukin-1 β (IL1 β) or RIP, a mediator of the TNF α signaling pathway (Huang *et al.*, [1997] *Supra*; Hsu *et al.*, [1996], *Supra*). As expected, stimulation of the IL1R and expression of RIP induced NF- κ B activation in the absence of LPS (Fig. 8B). Significantly, LPS did not enhance NF- κ B activation induced by IL1R stimulation or RIP (Fig. 8B).

Plant disease-resistant proteins have C-terminal LRRs that are critical for pathogen-specific responses (Dixon *et al.*, [2000], *Supra*). Alterations in their LRRs results in unresponsiveness to particular pathogens (Dixon *et al.*, [2000], *Supra*), suggesting that the LRRs of Nod1 might be also required for the response to LPS. To test this hypothesis, HEK293T cells were transfected with plasmids expressing wild-type or truncated Nod1 mutant lacking the LRRs (Nod1 Δ LRR) and treated with LPS. Expression of Nod1 Δ LRR induced higher NF- κ B activation than wild-type Nod1 in the absence of LPS, as previously reported (Inohara *et al.*, [1999], *Supra*). Significantly, LPS did not enhance NF- κ B activation induced by Nod1 Δ LRR (Fig. 9). Thus, the LRRs are essential for Nod1 to respond to LPS.

Several studies have provided conclusive evidence that TLR4 is a cell surface receptor for LPS (Aderam and Ulevitch, *Supra*, Poltorak *et al.*, Science, 282:2085 [1998]; Chow *et al.*, *Supra*; Takeuchi *et al.*, Immunity, 4:443 [1999]). Therefore, it is possible that expression of Nod1 confers LPS responsiveness through TLR4. To test this possibility, HEK293T cells were co-transfected with a TLR4 expression plasmid and NF- κ B activity was measured in the presence and absence of LPS. Expression of TLR4 alone did not induce NF- κ B activation in the presence of LPS, which is consistent with recent reports that additional cell surface molecules such as MD2 and CD14 are required for TLR4-mediated LPS responses in cells (Chow *et al.*, *Supra*; Takeuchi *et al.*, *Supra*). In accord with the latter, co-transfection of TLR4, CD14 and MD2 expression plasmids induced 8-fold activation of NF- κ B (see figure legend of Fig. 8C). To further verify that Nod1 confers LPS responsiveness independently of TLRs, a dominant negative mutant of MyD88, a common signaling molecule of IL-1

and Toll-related receptors including TLR4, was co-expressed with Nod1 or TLR4, CD14 and MD2 as a control, and transfected cells were stimulated with LPS. Co-expression of the MyD88 mutant suppressed NF- κ B activation induced by both TLR4 and ILIR stimulation, but it did not affect LPS-mediated NF- κ B activation induced by Nod1 (Fig. 8C). Furthermore, expression of a dominant negative mutant of TRAF6, a signaling molecule of TLR signaling pathways, did not block NF- κ B activation induced by Nod1, but inhibited TLR4-mediated NF- κ B activation (Inohara *et al.*, [1999], *Supra*). These results indicate that NF- κ B activation in Nod1-expressing cells induced by LPS is not mediated by the TLR4 signaling pathway. Consistent with this notion are recent observations showing that the Nod1 signaling pathway leading to NF- κ B activation is distinct to that of TLRs. Nod1 activates NF- κ B through its association with RICK, a protein kinase that directly interacts with IKK γ /NEMO, the regulatory subunit of the I κ B kinase complex (Inohara *et al.*, [2000], *Supra*).

LPS from different gram-negative bacteria have diverse structures (Rietschel *et al.*, Curr Top. Microbiol. Immunol., 216:39-81 [1997]). To determine if Nod1 confers responsiveness to LPS from several bacterial sources, Nod1-expressing cells were stimulated with LPS from six pathogenic bacteria or TNF α , as a positive control. All LPS preparations induced NF- κ B activation in Nod1-expressing cells, but different sources of LPS differed in their ability to enhance Nod1-mediated NF- κ B activation (Fig. 9). As it was found with LPS from *Escherichia coli* 055:B5 (Fig. 8B), none of the LPS preparations induced significant NF- κ B activation in cells expressing a Nod1 mutant lacking the LRRs (Fig. 9).

Plants have numerous disease resistant R genes and mammalian as well as insect cells have multiple TLR family members to respond to different pathogens (Dixon *et al.*, [2000], *supra*). Notably, Nod2, another Nod1-like protein that is homologous to Nod1 (34% amino acid identity) is comprised of N-terminal CARDS, NBD and LRRs. The presence of multiple Nod family members suggests that Nod1 and Nod2 may have different specificities for pathogen components. To test this, HEK293T cells were co-transfected with plasmids expressing wild-type or mutant

Nod2 lacking the LRRs. As it was observed with Nod1, all LPS preparations including those from invasive bacteria such as *Salmonella* and *Shigella*, stimulated NF- κ B activation in cells expressing wild-type Nod2 but not mutant Nod2 (Fig. 9). Notably, LPS from *Serratia marcescens* and *Salmonella typhimurium* was more effective in inducing NF- κ B activation in cells expressing Nod2 than Nod1 (Fig. 9). Furthermore, PGN preparation from *Staphylococcus aureus* stimulated NF- κ B activation in cells expressing Nod2 but not Nod1 (Fig. 9). The molecular basis for the differential response of Nod1 and Nod2 to both LPS and PGN is unclear. Further biochemical analyses and structure determination of LPS moiety recognized by Nod1 and Nod2 are required to understand the differential response of Nod proteins to bacterial components.

Apaf-1, a Nod1-like molecule which plays a central role in apoptosis, mediates responsiveness to cytochrome c leaked from mitochondria (Dixon *et al.*, *Supra*; Li *et al.*, *Cell*, 91:479-489 [1997]). Apaf-1 directly binds to cytochrome c (Li *et al.*, *Supra*). To determine if Nod1 binds to LPS, S100 cell lysates were prepared from HEK293T cells expressing Nod1 and the ability of Nod1 to bind radiolabeled LPS was tested by a modified immunoprecipitation assay. LPS was co-immunoprecipitated with Flag-tagged Nod1, but not with other Flag-tagged control proteins (Fig. 10A). Thus, Nod1 is associated with an LPS binding activity present in the cytosolic fraction of HEK293T cells. However, it is possible that Nod1 does not directly bind to LPS and that the association requires other cytosolic factors. For example, dATP or ATP is required for the response of Apaf-1 to cytochrome c (Li *et al.*, *Supra*). To begin to test this, we first immunoprecipitated Nod1 or IKK β , as a control protein, with anti-Flag antibody and the ability of the immunoprecipitated proteins to bind LPS was tested in nucleotide-free buffer. Immunopurified Nod1 exhibited LPS binding activity, but control IKK β did not (Fig. 10). These results suggest that Nod1 directly binds LPS. However, the possibility can not be excluded that Nod1 interacts with LPS through an intrinsic cytosolic factor(s) that is tightly bound to Nod1 and co-immunoprecipitates with Nod1 in the absence of LPS. In plants, the *Arabidopsis*

thaliana disease resistance RPS2 gene product that is structurally related to Nod1 and Nod2 can form a protein complex *in vivo* with the product of the phytopathogenic bacterium *Pseudomonas syringae* avrRpt2 gene but the protein complex also contained at least one additional plant protein of approximately 75 kDa (Leister and Katagiri, Plant J., 22:345-354 [2000]).

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology, genetics, or related fields are intended to be within the scope of the following claims.

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	Asn Tyr Ile Thr Ala Ala Gly Ala Gln Val Leu Ala Glu Gly Leu Arg		
		885	890 895
	Gly Asn Thr Ser Leu Gln Phe Leu Gly Phe Trp Gly Asn Arg Val Gly		
		900	905 910
10	Asp Glu Gly Ala Gln Ala Leu Ala Glu Ala Leu Gly Asp His Gln Ser		
		915	920 925
	Leu Arg Trp Leu Ser Leu Val Gly Asn Asn Ile Gly Ser Val Gly Ala		
		930	935 940
15	Gln Ala Leu Ala Leu Met Leu Ala Lys Asn Val Met Leu Glu Glu Leu		
		945	950 955 960
	Cys Leu Glu Glu Asn His Leu Gln Asp Glu Gly Val Cys Ser Leu Ala		
		965	970 975
	Glu Gly Leu Lys Lys Asn Ser Ser Leu Lys Ile Leu Lys Leu Ser Asn		
		980	985 990
20	Asn Cys Ile Thr Tyr Leu Gly Ala Glu Ala Leu Leu Gln Ala Leu Glu		
		995	1000 1005
	Arg Asn Asp Thr Ile Leu Glu Val Trp Leu Arg Gly Asn Thr Phe		
		1010	1015 1020
25	Ser Leu Glu Glu Val Asp Lys Leu Gly Cys Arg Asp Thr Arg Leu		
		1025	1030 1035
	Leu Leu		
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15

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25

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Ala Ile Val Arg Arg Leu His Ser His Val Glu Asn Met Leu Asp Leu
115 120 125

Leu Pro Ile Phe Thr Pro Ser Gln Arg Ala Arg Arg Leu Leu Asp Leu
145 150 155 160

Ala Thr Val Lys Ala Asn Gly Leu Ala Ala Phe Leu Leu Gln His Val
165 170 175

Gln Glu Leu Pro Val Pro Leu Ala Leu Pro Leu Glu Ala Ala Thr Cys
180 185 190

Lys Lys Tyr Met Ala Lys Leu Arg Thr Thr Val Ser Ala Gln Ser Arg
195 200 205

Phe Leu Ser Thr Tyr Asp Gly Ala Glu Thr Leu Cys Leu Glu Asp Ile
210 215 220

Tyr Thr Glu Asn Val Leu Glu Val Trp Ala Asp Val Gly Met Ala Gly
225 230 235 240

	Pro	Pro	Gln	Lys	Ser	Pro	Ala	Thr	Leu	Gly	Leu	Glu	Glu	Leu	Phe	Ser
					245					250					255	
	Thr	Pro	Gly	His	Leu	Asn	Asp	Asp	Ala	Asp	Thr	Val	Leu	Val	Val	Gly
				260					265					270		
5	Glu	Ala	Gly	Ser	Gly	Lys	Ser	Thr	Leu	Leu	Gln	Arg	Leu	His	Leu	Leu
			275					280					285			
	Trp	Ala	Ala	Gly	Gln	Asp	Phe	Gln	Glu	Phe	Leu	Phe	Val	Phe	Pro	Phe
		290					295					300				
10	Ser	Cys	Arg	Gln	Leu	Gln	Cys	Met	Ala	Lys	Pro	Leu	Ser	Val	Arg	Thr
	305					310					315					320
	Leu	Leu	Phe	Glu	His	Cys	Cys	Trp	Pro	Asp	Val	Gly	Gln	Glu	Asp	Ile
					325					330					335	
	Phe	Gln	Leu	Leu	Leu	Asp	His	Pro	Asp	Arg	Val	Leu	Leu	Thr	Phe	Asp
				340					345					350		
15	Gly	Phe	Asp	Glu	Phe	Lys	Phe	Arg	Phe	Thr	Asp	Arg	Glu	Arg	His	Cys
			355					360					365			
	Ser	Pro	Thr	Asp	Pro	Thr	Ser	Val	Gln	Thr	Leu	Leu	Phe	Asn	Leu	Leu
		370					375					380				
20	Gln	Gly	Asn	Leu	Leu	Lys	Asn	Ala	Arg	Lys	Val	Val	Thr	Ser	Arg	Pro
	385					390					395					400
	Ala	Ala	Val	Ser	Ala	Phe	Leu	Arg	Lys	Tyr	Ile	Arg	Thr	Glu	Phe	Asn
					405					410					415	
	Leu	Lys	Gly	Phe	Ser	Glu	Gln	Gly	Ile	Glu	Leu	Tyr	Leu	Arg	Lys	Arg
				420					425					430		
25	His	His	Glu	Pro	Gly	Val	Ala	Asp	Arg	Leu	Ile	Arg	Leu	Leu	Gln	Glu
			435					440					445			
	Thr	Ser	Ala	Leu	His	Gly	Leu	Cys	His	Leu	Pro	Val	Phe	Ser	Trp	Met
		450					455					460				
30	Val	Ser	Lys	Cys	His	Gln	Glu	Leu	Leu	Leu	Gln	Glu	Gly	Gly	Ser	Pro
	465					470					475					480
	Lys	Thr	Thr	Thr	Asp	Met	Tyr	Leu	Leu	Ile	Leu	Gln	His	Phe	Leu	Leu

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	485	490	495
	His Ala Thr Pro 500	Pro Asp Ser Ala 505	Ser Gln Gly Leu Gly 510
5	Leu Arg Gly 515	Arg Leu Pro Thr 520	Leu His Leu Gly 525
	Trp Gly 530	Leu Gly Met Cys 535	Tyr Val Phe Ser 540
	Ala Ala Gln Val 545	Ser Pro Asp Asp 550	Ile Ser Leu Gly 555
10	Ala Lys Gly Val 565	Pro Gly Ser Thr 570	Ala Pro Leu Glu 575
	Ile Thr Phe 580	Gln Cys Phe Phe 585	Ala Phe Tyr Leu 590
15	Asp Val Pro 595	Pro Ala Leu Leu 600	Arg His Leu Phe 605
	Gly Asn Ser 610	Pro Met Ala Arg 615	Leu Leu Pro Thr 620
	Ser Glu Gly 625	Lys Asp Ser Ser 630	Val Ala Ala Leu 635
20	Pro His Asn 645	Leu Gln Ile Thr 645	Ala Ala Phe Leu 650
	Arg Glu His 660	Trp Gly Leu Leu 665	Ala Glu Cys Gln 670
25	Leu Leu Arg 675	Arg Gln Ala Cys 680	Ala Arg Trp Cys 685
	Arg Lys His 690	Phe His Ser Ile 695	Pro Pro Ala Ala 700
	Ser Val His 705	Ala Met Pro Gly 710	Phe Ile Trp Leu 715
30	Glu Met Gln 725	Glu Arg Leu Ala 730	Arg Lys Ala Ala 735

	Val Gly His Leu Lys Leu Thr Phe Cys Ser Val Gly Pro Thr Glu Cys	740	745	750
	Ala Ala Leu Ala Phe Val Leu Gln His Leu Arg Arg Pro Val Ala Leu	755	760	765
5	Gln Leu Asp Tyr Asn Ser Val Gly Asp Ile Gly Val Glu Gln Leu Leu	770	775	780
	Pro Cys Leu Gly Val Cys Lys Ala Leu Tyr Leu Arg Asp Asn Asn Ile	785	790	795
10	Ser Asp Arg Gly Ile Cys Lys Leu Ile Glu Cys Ala Leu His Cys Glu	805	810	815
	Gln Leu Gln Lys Leu Ala Leu Phe Asn Asn Lys Leu Thr Asp Gly Cys	820	825	830
	Ala His Ser Met Ala Lys Leu Leu Ala Cys Arg Gln Asn Phe Leu Ala	835	840	845
15	Leu Arg Leu Gly Asn Asn Tyr Ile Thr Ala Ala Gly Ala Gln Val Leu	850	855	860
	Ala Glu Gly Leu Arg Gly Asn Thr Ser Leu Gln Phe Leu Gly Phe Trp	865	870	875
20	Gly Asn Arg Val Gly Asp Glu Gly Ala Gln Ala Leu Ala Glu Ala Leu	885	890	895
	Gly Asp His Gln Ser Leu Arg Trp Leu Ser Leu Val Gly Asn Asn Ile	900	905	910
	Gly Ser Val Gly Ala Gln Ala Leu Ala Leu Met Leu Ala Lys Asn Val	915	920	925
25	Met Leu Glu Glu Leu Cys Leu Glu Glu Asn His Leu Gln Asp Glu Gly	930	935	940
	Val Cys Ser Leu Ala Glu Gly Leu Lys Lys Asn Ser Ser Leu Lys Ile	945	950	955
30	Leu Lys Leu Ser Asn Asn Cys Ile Thr Tyr Leu Gly Ala Glu Ala Leu	965	970	975
	Leu Gln Ala Leu Glu Arg Asn Asp Thr Ile Leu Glu Val Trp Leu Arg	980	985	990

Gly Asn Thr Phe Ser Leu Glu Glu Val Asp Lys Leu Gly Cys Arg Asp
 995 1000 1005

Thr Arg Leu Leu Leu
 1010

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Met Gly Glu Glu Gly Gly Ser Ala Ser His Asp Glu Glu Glu Arg Ala
 1 5 10 15

Ser Val Leu Leu Gly His Ser Pro Gly Cys Glu Met Cys Ser Gln Glu
 20 25 30

Ala Phe Gln Ala Gln Arg Ser Gln Leu Val Glu Leu Leu Val Ser Gly
 35 40 45

Ser Leu Glu Gly Phe Glu Ser Val Leu Asp Trp Leu Leu Ser Trp Glu
 50 55 60

Val Leu Ser Trp Glu Asp Tyr Glu Gly Phe His Leu Leu Gly Gln Pro
 65 70 75 80

Leu Ser His Leu Ala Arg Arg Leu Leu Asp Thr Val Trp Asn Lys Gly
 85 90 95

Thr Trp Ala Cys Gln Lys Leu Ile Ala Ala Ala Gln Glu Ala Gln Ala
 100 105 110

Asp Ser Gln Ser Pro Lys Leu His Gly Cys Trp Asp Pro His Ser Leu
 115 120 125

His Pro Ala Arg Asp Leu Gln Ser His Arg Pro Ala Ile Val Arg Arg
 130 135 140

Leu His Ser His Val Glu Asn Met Leu Asp Leu Ala Trp Glu Arg Gly
 145 150 155 160

Phe Val Ser Gln Tyr Glu Cys Asp Glu Ile Arg Leu Pro Ile Phe Thr

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	165	170	175
	Pro Ser Gln Arg Ala Arg Arg Leu Leu Asp Leu Ala Thr Val Lys Ala 180 185 190		
5	Asn Gly Leu Ala Ala Phe Leu Leu Gln His Val Gln Glu Leu Pro Val 195 200 205		
	Pro Leu Ala Leu Pro Leu Glu Ala Ala Thr Cys Lys Lys Tyr Met Ala 210 215 220		
	Lys Leu Arg Thr Thr Val Ser Ala Gln Ser Arg Phe Leu Ser Thr Tyr 225 230 235 240		
10	Asp Gly Ala Glu Thr Leu Cys Leu Glu Asp Ile Tyr Thr Glu Asn Val 245 250 255		
	Leu Glu Val Trp Ala Asp Val Gly Met Ala Gly Pro Pro Gln Lys Ser 260 265 270		
15	Pro Ala Thr Leu Gly Leu Glu Glu Leu Phe Ser Thr Pro Gly His Leu 275 280 285		
	Asn Asp Asp Ala Asp Thr Val Leu Val Val Gly Glu Ala Gly Ser Gly 290 295 300		
	Lys Ser Thr Leu Leu Gln Arg Leu His Leu Leu Trp Ala Ala Gly Gln 305 310 315 320		
20	Asp Phe Gln Glu Phe Leu Phe Val Phe Pro Phe Ser Cys Arg Gln Leu 325 330 335		
	Gln Cys Met Ala Lys Pro Leu Ser Val Arg Thr Leu Leu Phe Glu His 340 345 350		
25	Cys Cys Trp Pro Asp Val Gly Gln Glu Asp Ile Phe Gln Leu Leu Leu 355 360 365		
	Asp His Pro Asp Arg Val Leu Leu Thr Phe Asp Gly Phe Asp Glu Phe 370 375 380		
	Lys Phe Arg Phe Thr Asp Arg Glu Arg His Cys Ser Pro Thr Asp Pro 385 390 395 400		
30	Thr Ser Val Gln Thr Leu Leu Phe Asn Leu Leu Gln Gly Asn Leu Leu 405 410 415		

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	Lys	Asn	Ala	Arg	Lys	Val	Val	Thr	Ser	Arg	Pro	Ala	Ala	Val	Ser	Ala	
				420					425					430			
	Phe	Leu	Arg	Lys	Tyr	Ile	Arg	Thr	Glu	Phe	Asn	Leu	Lys	Gly	Phe	Ser	
			435					440					445				
5	Glu	Gln	Gly	Ile	Glu	Leu	Tyr	Leu	Arg	Lys	Arg	His	His	Glu	Pro	Gly	
		450					455					460					
	Val	Ala	Asp	Arg	Leu	Ile	Arg	Leu	Leu	Gln	Glu	Thr	Ser	Ala	Leu	His	
	465					470					475					480	
10	Gly	Leu	Cys	His	Leu	Pro	Val	Phe	Ser	Trp	Met	Val	Ser	Lys	Cys	His	
					485					490					495		
	Gln	Glu	Leu	Leu	Leu	Gln	Glu	Gly	Gly	Ser	Pro	Lys	Thr	Thr	Thr	Asp	
				500					505					510			
	Met	Tyr	Leu	Leu	Ile	Leu	Gln	His	Phe	Leu	Leu	His	Ala	Thr	Pro	Pro	
			515					520					525				
15	Asp	Ser	Ala	Ser	Gln	Gly	Leu	Gly	Pro	Ser	Leu	Leu	Arg	Gly	Arg	Leu	
		530					535					540					
	Pro	Thr	Leu	Leu	His	Leu	Gly	Arg	Leu	Ala	Leu	Trp	Gly	Leu	Gly	Met	
	545					550					555					560	
20	Cys	Cys	Tyr	Val	Phe	Ser	Ala	Gln	Gln	Leu	Gln	Ala	Ala	Gln	Val	Ser	
					565					570					575		
	Pro	Asp	Asp	Ile	Ser	Leu	Gly	Phe	Leu	Val	Arg	Ala	Lys	Gly	Val	Val	
				580					585					590			
	Pro	Gly	Ser	Thr	Ala	Pro	Leu	Glu	Phe	Leu	His	Ile	Thr	Phe	Gln	Cys	
			595					600					605				
25	Phe	Phe	Ala	Ala	Phe	Tyr	Leu	Ala	Leu	Ser	Ala	Asp	Val	Pro	Pro	Ala	
		610					615					620					
	Leu	Leu	Arg	His	Leu	Phe	Asn	Cys	Gly	Arg	Pro	Gly	Asn	Ser	Pro	Met	
	625					630					635					640	
30	Ala	Arg	Leu	Leu	Pro	Thr	Met	Cys	Ile	Gln	Ala	Ser	Glu	Gly	Lys	Asp	
					645					650					655		
	Ser	Ser	Val	Ala	Ala	Leu	Leu	Gln	Lys	Ala	Glu	Pro	His	Asn	Leu	Gln	
				660					665					670			

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	Ile	Thr	Ala	Ala	Phe	Leu	Ala	Gly	Leu	Leu	Ser	Arg	Glu	His	Trp	Gly	
			675					680					685				
	Leu	Leu	Ala	Glu	Cys	Gln	Thr	Ser	Glu	Lys	Ala	Leu	Leu	Arg	His	Gln	
			690				695					700					
5	Ala	Cys	Ala	Arg	Trp	Cys	Leu	Ala	Arg	Ser	Leu	Arg	Lys	His	Phe	His	
	705					710					715					720	
	Ser	Ile	Pro	Pro	Ala	Ala	Pro	Gly	Glu	Ala	Lys	Ser	Val	His	Ala	Met	
					725					730					735		
10	Pro	Gly	Phe	Ile	Trp	Leu	Ile	Arg	Ser	Leu	Tyr	Glu	Met	Gln	Glu	Glu	
				740					745					750			
	Arg	Leu	Ala	Arg	Lys	Ala	Ala	Arg	Gly	Leu	Asn	Val	Gly	His	Leu	Lys	
			755					760					765				
	Leu	Thr	Phe	Cys	Ser	Val	Gly	Pro	Thr	Glu	Cys	Ala	Ala	Leu	Ala	Phe	
		770					775					780					
15	Val	Leu	Gln	His	Leu	Arg	Arg	Pro	Val	Ala	Leu	Gln	Leu	Asp	Tyr	Asn	
	785					790					795					800	
	Ser	Val	Gly	Asp	Ile	Gly	Val	Glu	Gln	Leu	Leu	Pro	Cys	Leu	Gly	Val	
					805					810					815		
20	Cys	Lys	Ala	Leu	Tyr	Leu	Arg	Asp	Asn	Asn	Ile	Ser	Asp	Arg	Gly	Ile	
				820					825					830			
	Cys	Lys	Leu	Ile	Glu	Cys	Ala	Leu	His	Cys	Glu	Gln	Leu	Gln	Lys	Leu	
			835					840					845				
	Ala	Leu	Phe	Asn	Asn	Lys	Leu	Thr	Asp	Gly	Cys	Ala	His	Ser	Met	Ala	
		850					855					860					
25	Lys	Leu	Leu	Ala	Cys	Arg	Gln	Asn	Phe	Leu	Ala	Leu	Arg	Leu	Gly	Asn	
	865					870					875					880	
	Asn	Tyr	Ile	Thr	Ala	Ala	Gly	Ala	Gln	Val	Leu	Ala	Glu	Gly	Leu	Arg	
					885					890					895		
30	Gly	Asn	Thr	Ser	Leu	Gln	Phe	Leu	Gly	Phe	Trp	Gly	Asn	Arg	Val	Gly	
				900					905					910			
	Asp	Glu	Gly	Ala	Gln	Ala	Leu	Ala	Glu	Ala	Leu	Gly	Asp	His	Gln	Ser	

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	Leu Arg Trp Leu Ser Leu Val Gly Asn Asn Ile Gly Ser Val Gly Ala				
	930		935		940
5	Gln Ala Leu Ala Leu Met Leu Ala Lys Asn Val Met Leu Glu Glu Leu				
	945		950		955 960
	Cys Leu Glu Glu Asn His Leu Gln Asp Glu Gly Val Cys Ser Leu Ala				
		965		970	975
	Glu Gly Leu Lys Lys Asn Ser Ser Leu Lys Ile Leu Lys Leu Ser Asn .				
		980		985	990
10	Asn Cys Ile Thr Tyr Leu Gly Ala Glu Ala Leu Leu Gln Ala Leu Glu				
		995		1000	1005
	Arg Asn Asp Thr Ile Leu Glu Val Trp Leu Arg Gly Asn Thr Phe				
		1010		1015	1020
15	Ser Leu Glu Glu Val Asp Lys Leu Gly Cys Arg Asp Thr Arg Leu				
		1025		1030	1035
	Leu Leu				
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25	Leu Leu Val Ser Gly Ser Leu Glu Gly Phe Glu Ser Val Leu Asp Trp				
		20		25	30
	Leu Leu Ser Trp Glu Val Leu Ser Trp Glu Asp Tyr Glu Gly Phe His				
		35		40	45
30	Leu Leu Gly Gln Pro Leu Ser His Leu Ala Arg Arg Leu Leu Asp Thr				
		50		55	60

Val Trp Asn Lys Gly Thr Trp Ala Cys Gln Lys Leu Ile Ala Ala Ala
65 70 75 80

Gln Glu Ala Gln Ala Asp Ser Gln Ser Pro Lys Leu His Gly Cys Trp
85 90 95

5 Asp

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Ser Leu His Pro Ala Arg Asp Leu Gln Ser His Arg Pro Ala Ile Val
1 5 10 15

Arg Arg Leu His Ser His Val Glu Asn Met Leu Asp Leu Ala Trp Glu
20 25 30

Arg Gly Phe Val Ser Gln Tyr Glu Cys Asp Glu Ile Arg Leu Pro Ile
35 40 45

Phe Thr Pro Ser Gln Arg Ala Arg Arg Leu Leu Asp Leu Ala Thr Val
50 55 60

Lys Ala Asn Gly Leu Ala Ala Phe Leu Leu Gln His Val Gln Glu Leu
65 70 75 80

Pro Val Pro Leu Ala Leu Pro Leu Glu Ala Ala Thr Cys Lys
85 90

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25 <211> 305

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<400> 7

Pro Ala Thr Leu Gly Leu Glu Glu Leu Phe Ser Thr Pro Gly His Leu
1 5 10 15

	Asn	Asp	Asp	Ala	Asp	Thr	Val	Leu	Val	Val	Gly	Glu	Ala	Gly	Ser	Gly
				20					25					30		
	Lys	Ser	Thr	Leu	Leu	Gln	Arg	Leu	His	Leu	Leu	Trp	Ala	Ala	Gly	Gln
			35					40					45			
5	Asp	Phe	Gln	Glu	Phe	Leu	Phe	Val	Phe	Pro	Phe	Ser	Cys	Arg	Gln	Leu
	50						55					60				
	Gln	Cys	Met	Ala	Lys	Pro	Leu	Ser	Val	Arg	Thr	Leu	Leu	Phe	Glu	His
	65					70					75				80	
10	Cys	Cys	Trp	Pro	Asp	Val	Gly	Gln	Glu	Asp	Ile	Phe	Gln	Leu	Leu	Leu
					85					90				95		
	Asp	His	Pro	Asp	Arg	Val	Leu	Leu	Thr	Phe	Asp	Gly	Phe	Asp	Glu	Phe
				100					105					110		
	Lys	Phe	Arg	Phe	Thr	Asp	Arg	Glu	Arg	His	Cys	Ser	Pro	Thr	Asp	Pro
			115					120					125			
15	Thr	Ser	Val	Gln	Thr	Leu	Leu	Phe	Asn	Leu	Leu	Gln	Gly	Asn	Leu	Leu
	130						135					140				
	Lys	Asn	Ala	Arg	Lys	Val	Val	Thr	Ser	Arg	Pro	Ala	Ala	Val	Ser	Ala
	145					150					155					160
20	Phe	Leu	Arg	Lys	Tyr	Ile	Arg	Thr	Glu	Phe	Asn	Leu	Lys	Gly	Phe	Ser
				165						170					175	
	Glu	Gln	Gly	Ile	Glu	Leu	Tyr	Leu	Arg	Lys	Arg	His	His	Glu	Pro	Gly
				180					185					190		
	Val	Ala	Asp	Arg	Leu	Ile	Arg	Leu	Leu	Gln	Glu	Thr	Ser	Ala	Leu	His
			195					200					205			
25	Gly	Leu	Cys	His	Leu	Pro	Val	Phe	Ser	Trp	Met	Val	Ser	Lys	Cys	His
	210						215					220				
	Gln	Glu	Leu	Leu	Leu	Gln	Glu	Gly	Gly	Ser	Pro	Lys	Thr	Thr	Thr	Asp
	225					230					235					240
30	Met	Tyr	Leu	Leu	Ile	Leu	Gln	His	Phe	Leu	Leu	His	Ala	Thr	Pro	Pro
					245					250					255	
	Asp	Ser	Ala	Ser	Gln	Gly	Leu	Gly	Pro	Ser	Leu	Leu	Arg	Gly	Arg	Leu

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20

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260

265

270

Pro Thr Leu Leu His Leu Gly Arg Leu Ala Leu Trp Gly Leu Gly Met
275 280 285

Cys Cys Tyr Val Phe Ser Ala Gln Gln Leu Gln Ala Ala Gln Val Ser
290 295 300

Pro
305

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<213> Homo sapiens

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Arg Ser Leu Tyr Glu Met Gln Glu Glu Arg Leu Ala Arg Lys Ala Ala
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Arg Gly Leu Asn Val Gly His Leu Lys Leu Thr Phe
20 25 /

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<213> Homo sapiens

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Cys Ser Val Gly Pro Thr Glu Cys Ala Ala Leu Ala Phe Val Leu Gln
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His Leu Arg Arg Pro Val Ala Leu Gln Leu Asp Tyr
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Asn Ser Val Gly Asp Ile Gly Val Glu Gln Leu Leu Pro Cys Leu Gly
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Val Cys Lys Ala Leu Tyr Leu Arg Asp
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<213> Homo sapiens

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Asn Asn Ile Ser Asp Arg Gly Ile Cys Lys Leu Ile Glu Cys Ala Leu
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His Cys Glu Gln Leu Gln Lys Leu Ala Leu Phe Asn
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Asn Lys Leu Thr Asp Gly Cys Ala His Ser Met Ala Lys Leu Leu Ala
1 5 10 15

Cys Arg Gln Asn Phe Leu Ala Leu Arg Leu Gly Asn
20 25

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<400> 13

Asn Tyr Ile Thr Ala Ala Gly Ala Gln Val Leu Ala Glu Gly Leu Arg
1 5 10 15

5

Gly Asn Thr Ser Leu Gln Phe Leu Gly Phe Trp Gly
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Asn Arg Val Gly Asp Glu Gly Ala Gln Ala Leu Ala Glu Ala Leu Gly
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Asp His Gln Ser Leu Arg Trp Leu Ser Leu Val Gly
20 25

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Asn Asn Ile Gly Ser Val Gly Ala Gln Ala Leu Ala Leu Met Leu Ala
1 5 10 15

Lys Asn Val Met Leu Glu Glu Leu Cys Leu Glu Glu
20 25

<210> 16

25

<211> 28

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<213> Homo sapiens

<400> 16

Asn His Leu Gln Asp Glu Gly Val Cys Ser Leu Ala Glu Gly Leu Lys
 1 5 10 15

Lys Asn Ser Ser Leu Lys Ile Leu Lys Leu Ser Asn
 20 25

5 <210> 17
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<400> 17

10 Asn Cys Ile Thr Tyr Leu Gly Ala Glu Ala Leu Leu Gln Ala Leu Glu
 1 5 10 15

Arg Asn Asp Thr Ile Leu Glu Val Trp Leu Arg Gly
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20 Glu Ala Gly Ser Gly Lys Ser
 1 5

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25 <400> 19

Leu Leu Thr Phe Asp
 1 5

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5 Glu Ser His Pro His Ile Gln Leu Leu Lys Ser Asn Arg Glu Leu Leu
1 5 10 15

Val Thr His Ile Arg Asn Thr Gln Cys Leu Val Asp Asn Leu Leu Lys
20 25 30

10 Asn Asp Tyr Phe Ser Ala Glu Asp Ala Glu Ile Val Cys Ala Cys Pro
35 40 45

Thr Gln Pro Asp Lys Val Arg Lys Ile Leu Asp Leu Val Gln Ser Lys
50 55 60

Gly Glu Glu Val Ser Glu Phe Phe Leu Tyr Leu Leu Gln Gln Leu Ala
65 70 75 80

15 Asp Ala Tyr Val Asp Leu Arg Pro Trp Leu Leu Glu
85 90

<210> 21

<211> 92

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<400> 21

Gly Ile Ala Gln Gln Trp Ile Gln Ser Lys Arg Glu Asp Ile Val Asn
1 5 10 15

25 Gln Met Thr Glu Ala Cys Leu Asn Gln Ser Leu Asp Ala Leu Leu Ser
20 25 30

Arg Asp Leu Ile Met Lys Glu Asp Tyr Glu Leu Val Ser Thr Lys Pro
35 40 45

Thr Arg Thr Ser Lys Val Arg Gln Leu Leu Asp Thr Thr Asp Ile Gln
50 55 60

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Gly Glu Glu Phe Ala Lys Val Ile Val Gln Lys Leu Lys Asp Asn Lys
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Gln Met Gly Leu Gln Pro Tyr Pro Glu Ile Leu Val
85 90

5 <210> 22
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<213> Homo sapiens

<400> 22

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Thr Leu Gln Ala Asp Ser Gly Leu Leu Leu Asp Ala Leu Val Ala Arg
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15 Gly Val Leu Thr Gly Pro Glu Tyr Glu Ala Leu Asp Ala Leu Pro Asp
35 40 45

Ala Glu Arg Arg Val Arg Arg Leu Leu Leu Leu Val Gln Ser Lys Gly
50 55 60

Glu Ala Ala Cys Gln Glu Leu Leu Arg Cys Ala Gln Gln Thr Val Ser
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20 Met Pro Asp Pro Ala Trp Asp Trp Gln His Val Gly Pro
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25 <213> Homo sapiens

<400> 23

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30 Gly Ala Glu Val Leu Val Glu Gly Leu Val Leu Gln Tyr Leu Tyr Gln
20 25 30

Glu Gly Ile Leu Thr Glu Asn His Ile Gln Glu Ile Asn Ala Gln Thr
35 40 45

Thr Gly Leu Arg Lys Thr Met Leu Leu Leu Asp Ile Leu Pro Ser Arg
50 55 60

5 Gly Pro Lys Ala Phe Asp Thr Phe Leu Asp Ser Leu Gln Glu Phe Pro
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10 <211> 91

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Asp Ile Ile Thr Leu Glu Met Arg Glu Leu Ile Gln Ala Lys Val Gly
35 40 45

20 Ser Phe Ser Gln Asn Val Glu Leu Leu Asn Leu Leu Pro Lys Arg Gly
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Pro Gln Ala Phe Asp Ala Phe Cys Glu Ala Leu Arg Glu Thr Lys Gln
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25 Gly His Leu Glu Asp Met Leu Leu Thr Thr Leu
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Ser Ser His Leu Lys Val Asp Glu Ile Leu Glu Val Leu Ile Ala Lys
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Gln Val Leu Asn Ser Asp Asn Gly Asp Met Ile Asn Ser Cys Gly Thr
35 40 45

Val Arg Glu Lys Arg Arg Glu Ile Val Lys Ala Val Gln Arg Arg Gly
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Asp Val Ala Phe Asp Ala Phe Tyr Asp Ala Leu Arg Ser Thr Gly His
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Glu Gly Leu Ala Glu Val Leu Glu Pro Leu Ala
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Ile His Asp Phe Glu Pro Arg Asp Ala Leu Thr Tyr Leu Glu Gly Lys
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Asn Ile Phe Thr Glu Asp His Ser Glu Leu Ile Ser Lys Met Ser Thr
35 40 45

25

Arg Leu Glu Arg Ile Ala Asn Phe Leu Arg Ile Tyr Arg Arg Gln Ala
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Ser Glu Leu Gly Pro Leu Ile Asp Phe Phe Asn Tyr Asn Asn Gln Ser
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30

His Leu Ala Asp Phe Leu Glu Asp Tyr Ile
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 35 40 45
 Gly Ser Arg Arg Asp Gln Ala Arg Gln Leu Ile Ile Asp Leu Glu Thr
 50 55 60
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 65 70 75 80
 Gly Gln Asp Met Leu Ala Ser Phe Leu Arg Thr Asn Arg
 85 90

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 Gly Phe Leu Thr Ile Ser Glu Glu Glu Lys Val Arg Asn Glu Pro Thr
 35 40 45
 30 Gln Gln Gln Arg Ala Ala Met Leu Ile Lys Met Ile Leu Lys Lys Asp
 50 55 60

Asn Asp Ser Tyr Val Ser Phe Tyr Asn Ala Leu Leu His Glu Gly Tyr
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Lys Asp Leu Ala Ala Leu Leu His Asp Gly Ile Pro Val
85 90

5 <210> 29
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<400> 29

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Phe Gln Gln Leu Thr Cys Val Leu Pro Ile Leu Asp Asn Leu Leu Lys
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Ala Asn Val Ile Asn Lys Gln Glu His Asp Ile Ile Lys Gln Lys Thr
35 40 45

Gln Ile Pro Leu Gln Ala Arg Glu Leu Ile Asp Thr Ile Trp Val Lys
50 55 60

Gly Asn Ala Ala Ala Asn Ile Phe Lys Asn Cys Leu Lys Glu Ile Asp
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Ser Thr Leu Tyr Lys Asn Leu Phe Val Asp Lys Asn
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25 <213> Homo sapiens

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Thr Thr Gly Ile Leu Asn Glu Gln Gly Glu Thr Ile Phe Ile Leu Gly

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	Met	Ala	Gly	Cys	Gly	Lys	Ser	Val	Leu	Ala	Ala	Glu	Ala	Val	Arg	Asp
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	His	Ser	Leu	Leu	Glu	Gly	Cys	Phe	Pro	Gly	Gly	Val	His	Trp	Val	Ser
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	Cys	Thr	Arg	Leu	Asp	Gln	Asp	Glu	Ser	Phe	Ser	Gln	Arg	Leu	Pro	Leu
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25	Asn	Ile	Glu	Glu	Ala	Lys	Asp	Arg	Leu	Arg	Ile	Leu	Met	Leu	Arg	Lys
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	His	Pro	Arg	Ser	Leu	Leu	Ile	Leu	Asp	Asp	Val	Trp	Asp	Ser	Trp	Val
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	Leu	Lys	Ala	Phe	Asp	Ser	Gln	Cys	Gln	Ile	Leu	Leu	Thr	Thr	Arg	Asp
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 10 Asn Lys Gln Phe Lys Arg Ile Arg Lys Ser Ser Ser Tyr Asp Tyr Glu
 225 230 235 240
 Ala Leu Asp Glu Ala Met Ser Ile Ser Val Glu Met Leu Arg Glu Asp
 245 250 255
 Ile Lys Asp Tyr Tyr Thr Asp Leu Ser Ile Leu Gln Lys Asp Val Lys
 260 265 270
 15 Val Pro Thr Lys Val Leu Cys Ile Leu Trp Asp Met Glu Thr Glu Glu
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5	Asp Ser Gly Thr Ala Pro Lys Ser Thr Phe Asp Leu Phe Thr Asp Ile 65 70 75 80		
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	Phe Phe Lys Ser Cys Glu Pro Lys Thr Phe Glu Lys Met Ala Gln Leu 210 215 220		
25	Asn Asn Lys Leu Glu Ser Arg Gly Leu Val Gly Val Glu Cys Ile Thr 225 230 235 240		
	Pro Tyr Ser Tyr Lys Ser Leu Ala Met Ala Leu Gln Arg Cys Val Glu 245 250 255		
	Val Leu Ser Asp Glu Asp Arg Ser Ala Leu Ala Phe Ala Val Val Met 260 265 270		
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5	Ser Leu Glu Gly Phe Glu Ser Val Leu Asp Trp Leu Leu Ser Trp Glu 50 55 60		
	Val Leu Ser Trp Glu Asp Tyr Glu Gly Phe His Leu Leu Gly Gln Pro 65 70 75 80		
	Leu Ser His Leu Ala Arg Arg Leu Leu Asp Thr Val Trp Asn Lys Gly 85 90 95		
10	Thr Trp Ala Cys Gln Lys Leu Ile Ala Ala Ala Gln Glu Ala Gln Ala 100 105 110		
	Asp Ser Gln Ser Pro Lys Leu His Gly Cys Trp Asp Pro His Ser Leu 115 120 125		
15	His Pro Ala Arg Asp Leu Gln Ser His Arg Pro Ala Ile Val Arg Arg 130 135 140		
	Leu His Ser His Val Glu Asn Met Leu Asp Leu Ala Trp Glu Arg Gly 145 150 155 160		
	Phe Val Ser Gln Tyr Glu Cys Asp Glu Ile Arg Leu Pro Ile Phe Thr 165 170 175		
20	Pro Ser Gln Arg Ala Arg Arg Leu Leu Asp Leu Ala Thr Val Lys Ala 180 185 190		
	Asn Gly Leu Ala Ala Phe Leu Leu Gln His Val Gln Glu Leu Pro Val 195 200 205		
25	Pro Leu Ala Leu Pro Leu Glu Ala Ala Thr Cys Lys Lys Tyr Met Ala 210 215 220		
	Lys Leu Arg Thr Thr Val Ser Ala Gln Ser Arg Phe Leu Ser Thr Tyr 225 230 235 240		
	Asp Gly Ala Glu Thr Leu Cys Leu Glu Asp Ile Tyr Thr Glu Asn Val 245 250 255		
30	Leu Glu Val Trp Ala Asp Val Gly Met Ala Gly Pro Pro Gln Lys Ser 260 265 270		

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